CHAPTER 2

Canine Distemper Virus as a Delivery Vector: Rescue of Recombinant CDV Expressing Dog Superoxide Dismutase 1 for Antioxidant Therapy

SUMMARY

Oxidative stress is thought to be a main cause of neurological disorders in mammals. Development of delivery system of the antioxidant enzymes to target cells is important for an effective therapy. In chapter 2, author constructed a recombinant CDV expressing dog superoxide dismutase 1 (SOD1) and characterized the effect of recombinant CDV. A recombinant virus, SOD1-CDV, was successfully rescued, which was confirmed to express SOD1 in a marmoset B cell line, B95a. The size of CPE caused by the recombinant CDV and its growth kinetics were comparatively similar to the parental strain. Western blot analysis revealed that the exogenous dog SOD1 (17kDa) was co-expressed with the endogenous marmoset SOD1 (19kDa) in the infected B95a cells. In nitroblue tetrazolium (NBT) assay, the increased SOD activity in SOD1-CDV infected cells was shown. These results indicate that SOD1-CDV is expected to produce functional SOD1 in infected neurons and the recombinant CDV is considered to be useful as a delivery viral vector.

INTRODUCTION

Progressive accumulation of oxidative damage and other types of neuropathology in a brain ultimately result in neuronal dysfunction and cognitive decline. Animal models, including rodents, nonhuman primates and canine, are useful for analysis of the mechanisms underlying pathological diseases and aging, and for development of therapeutics for those diseases. Old dog are widely used as a model of Alzheimer's disease and aging [Cummings 1993, Ruehl 1995, Cummings 1996, Papaioannou 2001, Head 2002]. Analyses of neurological congenital diseases such as epilepsy and myelopathy, are also important clinically. Use of canine as models to study brain diseases and aging has advantages such as: (1) canines share many of the same environmental conditions with humans; (2) canine can perform a sophisticated repertoire of complex cognitive behaviors; (3) the brain in aged canines shows many pathological changes common to humans; and (4) neuropathology is significantly associated with cognitive decline [Ruehl, 1995; Adams, 2000].

Superoxide dismutases (SODs) are the first reactive and most important antioxidant enzymes against reactive oxygen species, particularly superoxide anion radicals. At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been described [Zelko, 2002]. CuZn-SOD (SOD1) of SOD has Cu and Zn in their catalytic center and are localized to intracellar cytoplasmic compartments. Mn-SOD (SOD2) also localizes intracellular. EC-SOD is released as extracellular elements (SOD3). SOD1 has been found in cytoplasm, nuclear compartments and lysosomes of mammalian cells [Chang, 1988; Keller, 1991; Crapo, 1992, Liou, 1993]. SOD1 gene has been localized to chromosome 21 (region 21p22) in humans [Accession M24535], chromosome 1 (1q12-14) in bovine

species [Barendse, 1993], chromosome 16 (region 16B4-ter) in mouse and chromosome 13 (region CFA31) in dog [Green, 2002]. Human chromosome 21 has been studied intensely because of the association between Down's syndrome and trisomy 21. Although patients with Down's syndrome show a 50% increased in SOD1 activity due to higher levels of SOD1 protein, the role of this enzyme in pathology associated with this disease is still undefined. The increase dosage of SOD1 gene associates with some symptoms of Down's syndrome, such as the pathological abnormalities of tongue neuromuscular junctions [Yarom, 1987] but has no obvious implication in the development of the major symptoms [Groner, 1995]. On the other hand, more than 90% of different mutations in the SOD1 gene have been associated with amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease. This fatal disorder causes degradation of motor neurons in the corticospinal tracts and brain stem. Although only 20 % of patients with ALS and 10-15% with familial ALS have mutations in the SOD1 gene, the discovery of these mutations by Rosen viruses in 1993 provided the first molecular insight into the pathogenesis of this disease [Rosen, 1993]. Since this discovery, several theories have been proposed to explain the mechanism of motor neuron damage caused by mutations in the SOD1. One hypothesis is that mutations in the SOD1 gene may impair antioxidant enzyme activity that in turn could lead to accumulation of toxic superoxide anions. This theory was dismissed experimentally when SOD1 bearing the G93A mutation was overexpressed in mice, resulting in motor neuron disorder despite the elevated SOD1 activity. Moreover, complete inactivation of SOD1 in "knock-out" mice do not cause any motor neuron abnormalities [Aguzzi, 1994], although they exhibit increasing embryonic lethality and reduced fertility in females [Ho, 1998].

Antioxidant gene therapy had been already major methods to protect against neurological disorders experimentally [Imaizumi, 1990; Nakao, 1995; Barkats, 1997; Estevez, 2000; Sha, 2001; Kim, 2002; Solas, 2003; Kawamoto, 2004]. But the delivery system of antioxidant gene had been still on a way by many roots or vectors because of the tight brain protection, blood brain barrier (BBB). Viral vectors are expected to be one of the good tools to overcome BBB and reach the target cells in central nervous system by their kinetics. In the chapter 1, it was appeared that recombinant CDV has high infectivity and ability of persistence in brain cells, especially neurons. In chapter 2, author constructed a recombinant CDV expressing SOD1 and characterized it.

MATERIALS AND METHODS

Cells and viruses

293 cells (a human embryonal kidney cell line) were maintained in Dullbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). B95a cells (an Epstein-Barr virus transformed marmoset lymphoblastoid cell line) [Kobune, 1990] were maintained in RPMI1640 containing 5% FCS. The Yanaka strain of CDV [Gemma, 1996b] and rescued viruses were grown on B95a cells. The host range mutant of recombinant vaccinia virus Ankara (MVA-T7), which expresses T7 RNA polymerase, was a kind gift from Dr G. Shutter.

Plasmid constructions and virus rescue

Full-length SOD1 cDNA (462 bp) was obtained from a dog brain. Total RNA was extracted by dog brain with Isogene (Nippongene), and RT-PCR was performed with a forward primers: 5′-atggagatgaaggccgt-3′ and a reverse primer: 5′-ttattgggcgatcccaat-3′. After confirmed by sequencing, the cDNA was re-amplified by PCR using two new primers attached with FseI recognition sequence and CDV transcription unit at 5′ ends of the above primers [Fujita, 2002]. Amplified cDNA fragment was subcloned into TA cloning vector pCR (Invitrogen). The plasmid was digested by FseI and then the cDNA fragments were purified by QIAEX II (QIAGEN). They were inserted into the FseI site of a cDNA clone (pCDV), coding the genome of the Yanaka strain [Fujita, 2002]. The obtained cDNA clones, designated pSOD1-CDV, were applied for the virus rescue. The DNA sequences were confirmed by Big dye terminator and ABI3100 model sequencers (Applied Biosystem). For virus rescue, 293 cells were prepared at 70% confluency in 6-well plates with 10% FCS in DMEM. The cells were

infected with MVA-T7 at the MOI of 1 for 30 min. Following MVA-T7 infection, pSOD1-CDV was transfected into 293 cells with supporting plasmids (pRPV-N, pRPV-P and pRPV-L) as mixtures with Fugene 6 (Roche). After 3 days incubation at 37℃, the medium containing 2% FCS was removed and B95a cells ($1x10^6$ per well) were spread on 293 cells with RPMI1640 medium (Gibco, BRL). After the appearance of cytopathic effect (CPE), cells and supernatants were harvested and store at -80℃. For confirmation of rescued CDV, RT-PCR was performed using primers amplified between N and P sequence and the obtained PCR fragments were determined corresponding to SOD1 sequences.

Virus titration

Multistep growth kinetics were analyzed by infecting B95a cells with viruses at an MOI of 0.01 in 6-well plates. Virus was adsorbed to the cell for 1-2 hrs, and then inoculum removed, and 2ml of medium was supplied into each well. Virus induced CPE was visualized by infecting B95a cells at 48 hr post infection. At every 24 hr for 0 hr to 144 hr, the cells with medium were harvested and frozen at -80℃. The suspension was frozen and thawed three times and the released virus was determined by endpoint dilution assays to calculate 50% tissue culture infectious dose ($TCID_{50}$) values as standard method [Iwatsuki, 1995].

Western blot analysis

Infected cells were washed once with PBS and lysed on ice in lysis buffer containing PBS, 1% Triton X-100 in 10mM Tris-HCl (pH7.5)-5mN EDTA-1mM DTT-0.25mM phenylmethanelsulufonyl fluoride. The concentration of the total protein

was measured by BCA (PIERCE). Each sample (20μg/lane) was separated by 10%-SDS-polyacrylamid gel electrophoresis under reducing condition with 2-mercaptoethanol and then blotted onto Immobilon-P nylon membranes (Millipore). After blocked by PBS containing 4% block ace (Yukijirushi) and 0.05% Tween 20, the membranes were incubated in the antibody solution containing anti-human SOD1 antibodies immunized by rabbit (Stressgen) in PBS for 1 hr at room temperature. After washed, the membranes were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (CAPPEL). Labeled proteins were detected using the ECL plus western blotting detection system (Amersham Bioscience) and measured the intensity of protein band by LAS1000 (Fuji Film).

SOD enzymatic activity (NBT assay)

SOD activity was determined by gel electrophoresis followed by nitroblue tetrazolium (NBT) staining [Beauchamp, 1971]. Forty-eight hrs after infection, Nonidet P-40 extracts were prepared from SOD1-CDV infected or uninfected cultures. Cell extracts were loaded on a 15% non-denaturing polyacrylamide gel, and electrophoresis was performed at 100 V. SOD activity was revealed by soaking the gel in distilled water containing 0.3mM NBT and 0.26mM riboflavin (20 min, at room temperature) followed by incubation in 90mM tetramethyl-ethlenediamine (TEMED) for 20 min at room temperature.

RESULTS

Rescue of a recombinant SOD1-CDV

Dog SOD1 cDNA (462 bp) was obtained from a dog brain described in materials and methods. It encodes entirely 154 amino acids identical to previous report [Accession NM_001003035]. The entire open reading frame (ORF) was attached with a set of transcriptional end signal and start signal at the upstream of the ORF, and inserted between N and P genes (Fig. 1). The plasmids were applied for our standard CDV rescue system using 293 cells and MVA-T7, a recombinant vaccinia virus expressing T7 RNA polymerase [Fujita, 2002]. At 3 days after overlay of B95a cells on 293 cells, the typical cytopathic effect (CPE) was observed (Fig. 2A). The rescued virus was designated as the SOD1-CDV and was confirmed by RT-PCR using each set of specific primers, which encode the N gene and SOD1 gene (data not shown). In addition, the amplified DNA fragment was shown at an expected size and identical to SOD1 sequence.

Characterization of SOD1-CDV

After the SOD1-CDV was inoculated B95a cells, the CPE formation and virus growth kinetics were compared with its parental Yanaka strain. The size of CPE and growth kinetics by the recombinant CDV was almost similar to the parental strain (Fig 2A, B). The dog SOD1 mRNA was detected in SOD1-CDV infected B95a cells as well as that of MDCK cells by RT-PCR (Fig. 3). To further characterize the expression of the dog SOD1, B95a cells infected with SOD1-CDV were analyzed with immunofluorescence and western blot. Because the purchased antibody against human SOD1 has cross-reactivity to both marmoset and dog SOD1, uninfected B95a cells were also stained by immunofluorescent assay (data not shown). Molecular weight of SOD1 was

different among human (24 kDa), marmoset (19kDa) and dog (17kDa), and thus western blot analysis was performed by separating to soluble fraction and insoluble pellet (Fig. 4). Only endogenous marmoset SOD1 expression (19 kDa) was detected in CDV Yanaka infected B95a cells, while exogenous dog SOD1 expression was also observed in SOD1-CDV infected B95a cells. Interestingly, a large amount of dog SOD1 remained in insoluble pellet, probably indicating that dog SOD1 was overexpressed and aggregated in the infected cells.

Examination of dog SOD1 activity in B95a cells

To determine if SOD1-CDV could express a functional dog SOD1 protein in B95a cells, author investigated the level of SOD enzymatic activity in the cells at 3 dpi compared with uninfected cultures. Protein extracts from the cells were separated by native PAGE, and SOD activity was detected by NBT staining, which aimed to detect the reduction of NBT illumination by O_2^-, caused by inhibition of SODs. This assay, which is based on the determination of the level of superoxide quenching in the gel, allows a semiquantitative analysis of SOD enzymatic activity. As endogenous marmoset SOD and dog exogenous SOD could not be discriminated by their different mobilities in the native gel, total SOD activity was quantified in SOD1-CDV infected cells and compared to that of the control (CDV-Yanaka)-infected and uninfected cells (Fig. 5). Common reacted bands were detected among all cell extracts, and overexpression of dog SOD1 was identified in lysate of SOD1-CDV infected B95a cells. Thus, parental CDV Yanaka infection did not induce enhancement of SOD activity compared to uninfected B95a cells. The increased SOD activity in SOD1-CDV infected cells was thus due to the activity of the recombinant dog SOD1 protein.

DISCUSSION

In this study, author succeeded to express dog SOD1 abundantly using the recombinant CDV vector. In chapter 1, CDV is shown to be a promising vector for gene transfer in the central nervous systems, since the recombinant CDV allows expression of transgenes during a long term in neurons. Thus, this viral vector is efficient and useful for analysis of the certain neuroprotective proteins. Author appeared the possibility of recombinant CDV vectors as the therapeutic potential of CDV vectors containing dog superoxide dismutase 1 gene.

The intracellular SODs can be divided into two groups of proteins. The first group is comprised of SOD1 that are present in the cytoplasm of both eukaryotes and bacteria [Fridovich, 1974a, b, 1975; Beyer, 1991]. The second group is MnSOD and present normally within mitochondria [Bannister, 1987; Ho and Crapo, 1988; Beyer, 1991]. Both SODs protect cells from oxidative damage and regulate superoxide concentrations. It was reported that SOD1 was not effectively induced after MV [Fujinami, 1998] or influenza virus infection [Knobil, 1998]. This could explain the reason that SOD1-CDV infection did not induce or repress the expression of endogenous SOD1 in B95a cells. On the other hand, increased amounts of MnSOD are detectable in the serum of patients with acute viral infections, such as MV and influenza virus infections [Fujinami, 1998, Raoul, 1994, 1998; Knobil, 1998; Semrau, 1998]. In addition, MnSOD was up-regulated in B-cells during MV infection in vitro [Wang, 2003]. Taken together, it is speculated that total SODs are highly enhanced in SOD1-CDV infected cells due to expression of exogenous SOD1 and also up-regulation of endogenous MnSOD. Since growth kinetics of SOD1-CDV is similar to that of CDV-Yanaka, it is suggested that increase of SODs activity has little influence on CDV

replication.

Utility of SOD1 had been reported as a therapeutic enzyme against aging, heredity disease, brain injury, perfusion and cancer therapy [Oberley, 1979; Sagi, 1986; Imaizumi, 1990; Nakao, 1995; Barkats, 1997; Estevez, 2000; Sha, 2001; Kim, 2002; Solas, 2003; Kawamoto, 2004]. Hereditary canine spinal muscular atrophy (HCSMA), fatal inherited motor neuron disease in Brittany spaniels, shares many clinical and pathological features with human SOD1 mutational disease, familial amyotrophic lateral sclerosis (FALS) [Cork, 1982]. In dog's study of perfusion model, recombinant human SOD1 was used for trial to reducing the perfusion injury, which administrated intravenously. Dog senile dementia of Alzheimer type disease is caused by accumulation of β-amyloid in brain, which shares many symptoms with human Alzheimer [Skoumalova, 2003]. The evaluation of antioxidant diet treatment against dog Alzheimer had already reported and resulted in significant improvement [Cotman, 2002]. Author showed that SOD1-CDV produced functional SOD1 in infected cells. As would be expected from this study, SOD1-CDV might be useful for these diseases as antioxidant therapeutic viral vector.

FIGURE LEGENDS

Fig. 1 Construction of pSOD1-CDV. (A) Dog SOD1 cDNA was amplified by PCR using specific primers. (B) Scheme of pSOD1-CDV. The dog SOD1 cDNA was re-amplified by primers which contained viral transcription unit. The amplified cDNA was digested with FseI and inserted into FseI site between N and P gene of CDV.

Fig. 2 Characterization of SOD1-CDV. (A) SOD1-CDV infected B95a cells formed CPE as well as the parent the Yanaka strain. (B) Viral growth kinetics of the SOD1-CDV (■) compared with the Yanaka strain (▲). Both viruses were inoculated with B95a cells at MOI of 0.01. Both viruses were grown similarly and reached the similar maximal titer at 3 dpi.

Fig. 3 Expression of dog SOD1 mRNA. RT-PCR was performed by specific PCR primers coding dog SOD1 sequence. GAPDH DNA fragments were also amplified by PCR, showing the different mobilities between dog and marmoset SOD1. B95a cells were derived from marmoset B cell line and MDCK cells from dog kidney.

Fig. 4 Western blot analysis of dog SOD1 expression. Hela cells were derived from human endocervical carcinoma. The cell was lysed in lyses buffer and then the soluble supernatant and insoluble pellet were separated by centrifugation. SOD1s with different molecular weight of human (24 kDa), marmoset (19kDa) and dog (17kDa) were detected.

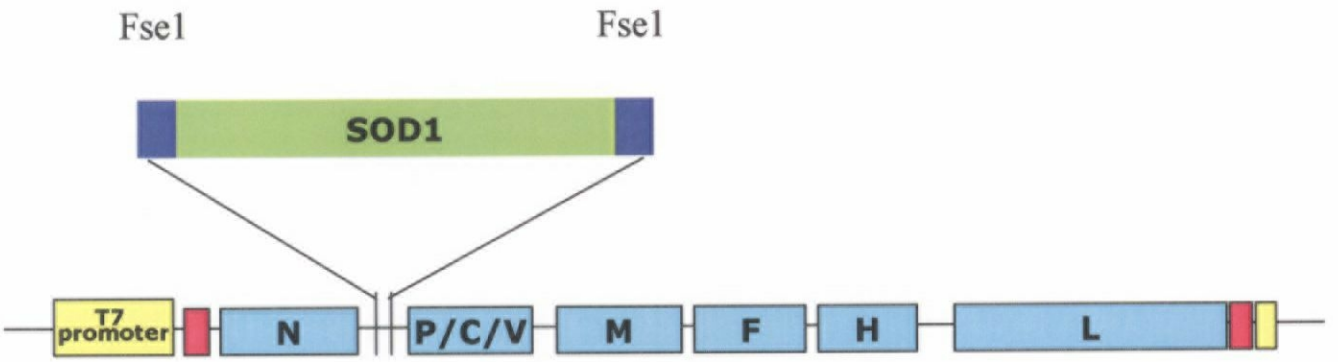
Fig. 5 Analysis of dog SOD1 activity by NBT assay. Forty-eight hrs after infection, Nonidet P-40 extracts were prepared from SOD1-CDV infected or uninfected cultures. Cell extracts were electrophoresed by native PAGE. SOD activity was revealed by soaking the gel in distilled water containing 0.3mM NBT and 0.26mM riboflavin. Bands show functional SOD1 proteins.

Fig. 1

A)

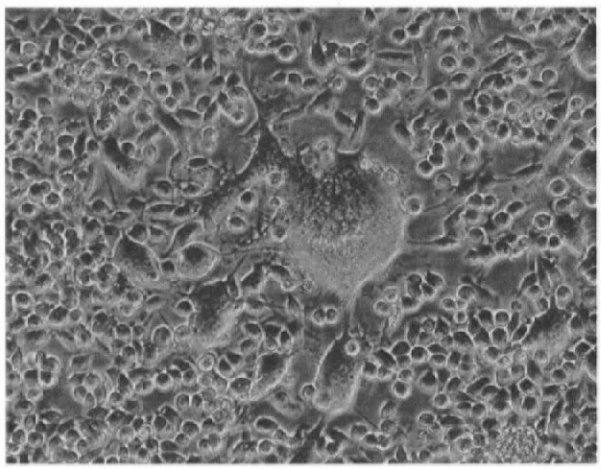
Dog SOD1 cDNA

B)

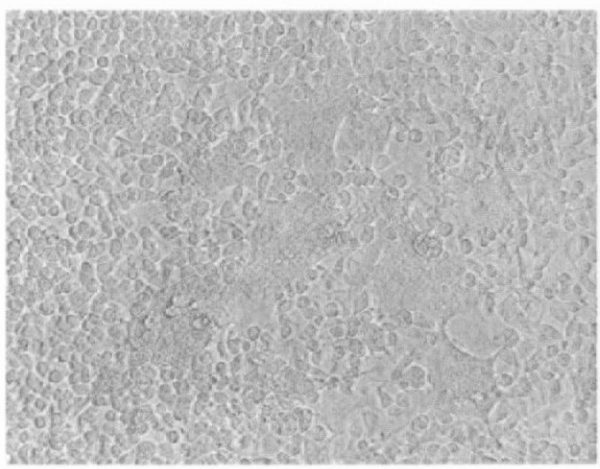


Full genome cDNA of CDV Yanaka in pMEDB1

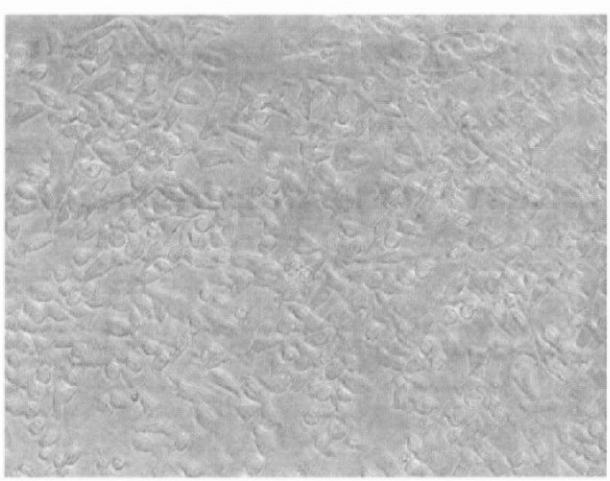
Fig. 2A



SOD1-CDV



Yanaka



B95a

Fig. 2B

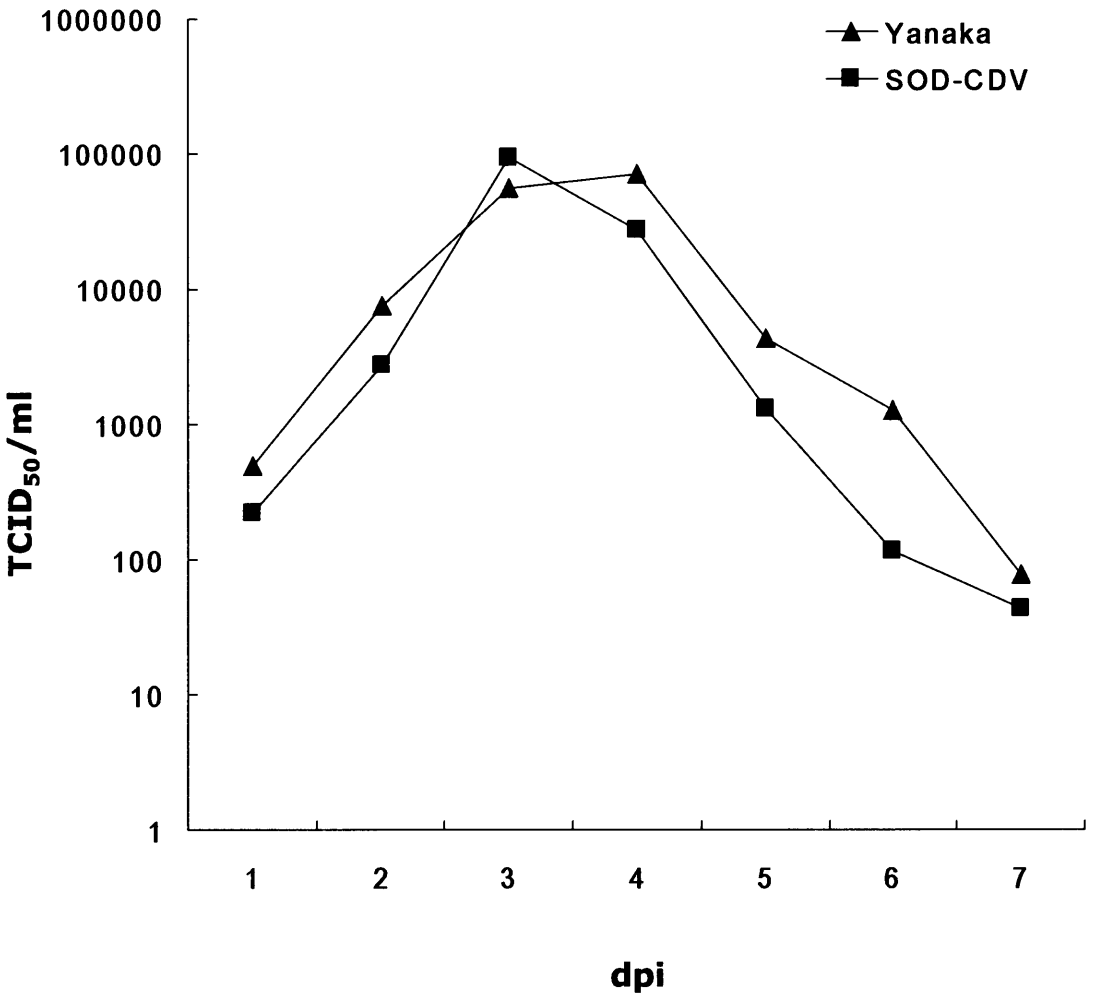

1000000
100000
10000
1000
100
10
1
TCID50/ml
Yanaka
SOD-CDV
1
2
3
4
5
6
7
dpi

Fig. 3

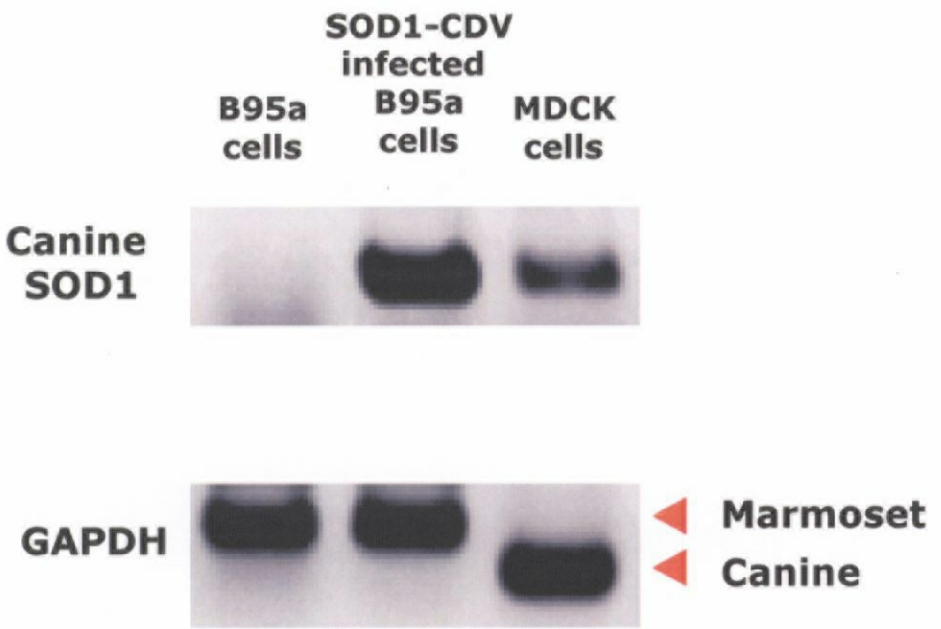

SOD1-CDV infected B95a cells
B95a cells
MDCK cells
Canine SOD1
GAPDH
Marmoset
Canine

Fig. 4

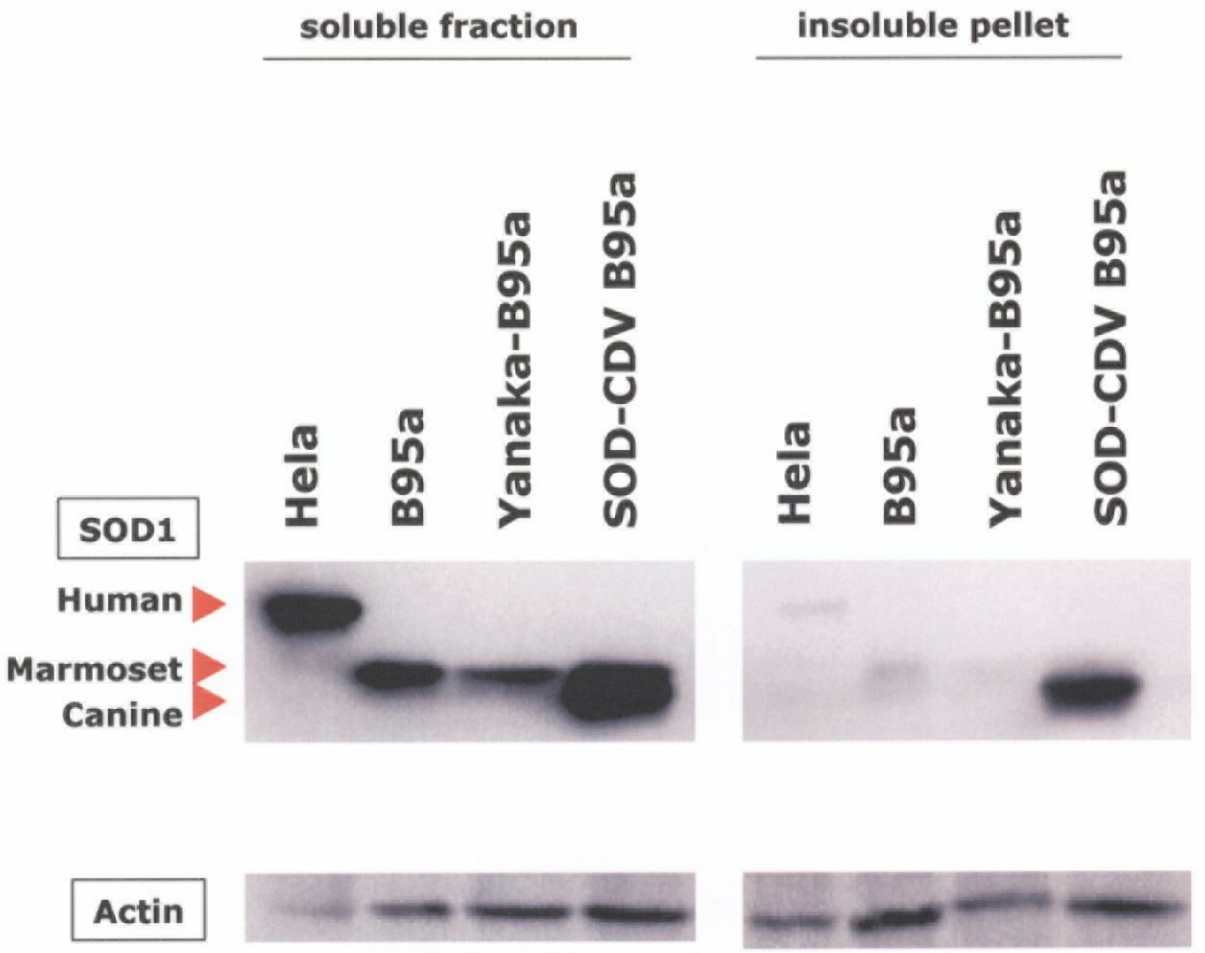
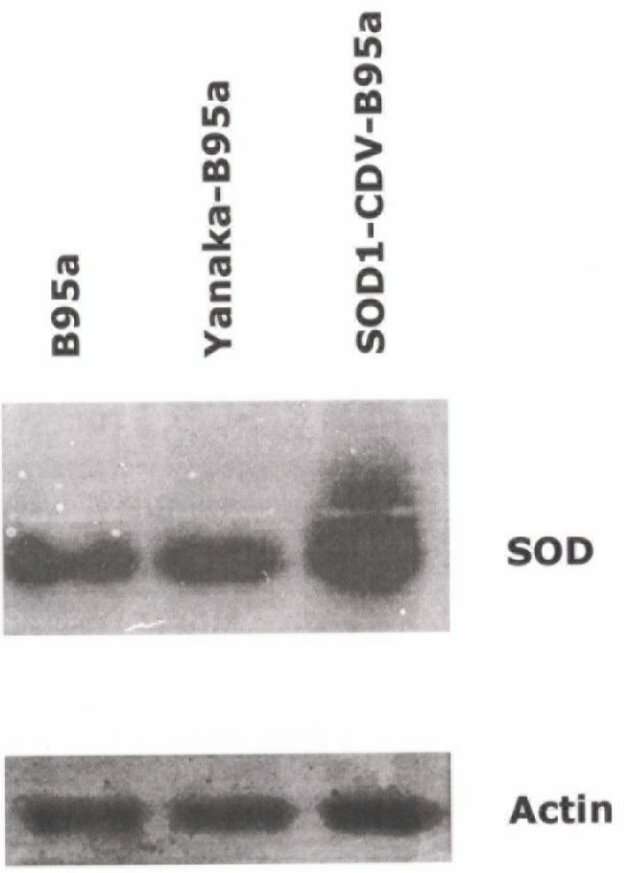

soluble fraction
insoluble pellet
Hela
B95a
Yanaka-B95a
SOD-CDV B95a
Hela
B95a
Yanaka-B95a
SOD-CDV B95a
SOD1
Human
Marmoset
Canine
Actin

Fig. 5



CHAPTER 3

Development of Polyvalent Canine Distemper Virus Vaccines against Canine Distemper Virus and Leishmania Infections

SUMMARY

Using reverse genetics of canine distemper virus (CDV), the author studied the availability of the recombinant CDV for the development of a polyvalent CDV vaccine against CDV and Leishmania infection. Leishmaniasis is one of the most serious parasitic zoonoses transmitted to humans from domestic dogs or rodents via infected sand flies. Effective vaccination of dogs against Leishmaniasis is expected to disrupt the infection cycle. The author constructed two recombinant CDVs expressing Leishmania antigen, a homologue to eukaryotic thiol-specific antioxidant protein (TSA) and a homologue of a yeast stress-inducible protein 1 (LmSTI1). Each full-length cDNA was inserted between the N and P genes of the infectious cDNA clone of the CDV Yanaka strain. The author successfully rescued the viruses, TSA-CDV and LmSTI1-CDV, which were confirmed to express the mRNAs and proteins in B95a cells. The recombinant CDVs and the parental strain reached approximately the same maximum titers at 72 to 96 hpi in B95a cells. Then dogs were inoculated with TSA-CDV or LmSTI1-CDV subcutaneously at $1x10^5$ $TCID_{50}$. The dogs did not show observable clinical signs of infection until 10 weeks post infection. High antibody titers (1024x-4096x dilution) against CDV were detected in all immunized dogs from 5 weeks post inoculation. However, antibodies against TSA or LmSTI1 were found quite low. Dogs immunized with the TSA-CDV, LmSTI1-CDV alone or the mixture of TSA-CDV, LmSTI1-CDV and LACK-CDV, which was previously established, and control dogs (n=2 each) were challenged with $5x10^7$ stationary-phase promastigotes of *L. major* intradermally in the ears between 5 to 8 weeks post immunization. Nodules appeared in the ears of control dogs at 10 days after the challenge and then enlarged. Most significant protective effects were found in the dogs inoculated with the mixture of TSA-CDV, LmSTI1-CDV

and LACK-CDV. The dogs developed apparently smaller nodules than the control dogs during all stage of infection after the challenge. The results indicate that the recombinant CDV with *Leishmania* antigens could induce protective immunity in the inoculated dogs against both CDV and *Leishmania* infections.

INTRODUCTION

Canine distemper (CD) is one of the lethal infectious diseases in dogs, raccoons, ferrets, seals, wild lions, etc. Canine distemper virus (CDV) induces fever, gastroenteritis, pneumonia, conjunctivitis and encephalomyelitis and showed high mortality in pappies. CDV is a member of the genus *Morbillivirus* in the family *Paramyxoviridae*. The incidence of CD in dogs was reduced after the live attenuated vaccines were developed and introduced from 1960s. However, CD outbreaks involving even vaccinated dogs have been observed worldwide from 1990s [Blixenkrone-Moller, 1992; Bolt, 1997; Ek-Kommonen, 1997]. Serum collected from vaccinated dogs showed comparatively lower neutralizing activity to the wild-type strains [Kai, 1993; Genma, 1996; Haas, 1997]. Iwatsuki and colleagues observed the genetical alterations between Hemagglutinin (H) gene of the vaccine strains and those of recent field isolates, which probably reflect the antigenic diversity [Iwatsuki, 1997]. These findings suggested that the present vaccines can give insufficient efficacy against current circulating wild-type CDVs. In 1997, Gemma and colleagues isolated recent wild type CDV, the Yanaka strain, which was an avilulent strain and considered to be a candidate for a noble vaccine strain [Gemma, 1996b; Takenaka in preparation]. Recently, we successfully constructed reverse genetics system of CDV with the strain [Fujita, 2002].

Leishmaniasis is distributed in parts of 88 countries with 12 million people in tropical and subtropical regions. Number of new cases of leishmaniasis each year in the world reaches more than one million. The World Health Organization documented in 1993 that Leishmaniasis was one of the six major tropical diseases of developing countries. Leishmaniasis is caused by infection with parasite protozoa *Leishmania* and a large spectrum of zoonotic diseases in human and animals, ranging from self-curing

skin ulcers to the severe and lethal pathologies of visceral form. Leishmaniasis is a complex disease with various symptoms, including cutaneous, mucocutaneous and visceral leishmaniasis. For the patients at the late stage, there are no effective treatment and drugs. The parasites are naturally transmitted by blood-sucking sand flies among reservoir animals including rodents and dogs. Humans are accidental hosts transmitted from the animals. In the previous reports, inducing cell-mediated immunity is more important to cure and protect from leishmaniasis than serological one [Sacks, 2002]. Epidemiologically, it was reported in Brazil that the elimination of canine leishmaniasis has been correlated with a decreased prevalence of disease in human [Dietze, 1997]. These are, however, no effective vaccines for human and even dogs [Vanloubbeeck, 2004]. Recently, the number of *L. major* (human leishmania) mutants are increasing in South Europe, which escape from anti leishmanial drugs [Medecins sans Frontieres, 2004] and one of the reasons is considered to be due to the treatment of the affected dogs with anti-leishmanial drugs repeatedly. Thus, domestic dogs are not only reservoirs of leishmania but also mutant leishmania producers.

In this chapter, to develop the vaccine targeted to *L. major*, author constructed recombinant CDVs with protective antigens of *Leishmania*, which antigens (TSA and LmSTI1) are expected to induce cell-mediated immunity as vaccine [Webb, 1996; Webb, 1998; Mougneau, 1995] and evaluated the availability of the recombinant CDVs.

MATERIALS AND METHODS

Cells, viruses and parasite

293 cells were maintained in Dullbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). B95a cells were maintained in RPMI1640 containing 5% FCS. The CDV Yanaka strain [Gemma, 1996b] and rescued viruses were grown in B95a cells. The recombinant vaccinia virus MVA-T7, which expresses T7 RNA polymerase, was a kind gift from Dr G. Shutter. Recombinant LACK-CDV was previously established [Miura, 2002]. *Leishmania major* (*L. major*) RM2 was described previously [Okuno, 2002] was used for challenge experiments. Promastigote of *L. major* was cultured in Schneider's medium (Gibco BRL) with 10% fetal carf serum (Sigma-Aldrich) and metacyclic stage of promastigote was used for protection assay.

Plasmid constructions and viral rescue

Full-length TSA and LmSTI1 were obtained from *L. major* [Webb, 1996; Webb, 1998]. Total RNA was extracted by promastigote of *L.* major with Isogene (Nippongene), and RT-PCR was performed with the following primers:

TSA: Forward (F) 5'-TGCACCATCACCATCACATGTCCT GCGGTAAC-3'

Reverse (R) 5'-ACGCTCACAGGTTTACTGCTTGCTGAAGTA-3'

LmST1: F 5'-GCACATTTCTCCGTAATGGACGCAACTGAG-3'

R 5'-CAGCGTAGAAGTCTACTGACCAAAACGAAT-3'

After confirmed by sequencing, the cDNAs were re-amplified by PCR using two new primers attached with FseI recognition sequence and CDV transcription unit at 5' ends of the above primers. Amplified cDNA fragments were subcloned into TA cloning vector pCR (Invitrogen). The plasmid was digested by FseI and the cDNA fragments were

purified by QIAEX II (QIAGEN). They were inserted into the FseI site of a cDNA clone, CDV, coding the genome of the Yanaka strain [Fujita, 2002]. The obtained cDNA clones, designated as pTSA-CDV and pLmSTI1-CDV were applied for the virus rescue. The DNA sequences were confirmed by Big dye terminator and ABI3100 Avant model sequencers (Applied Biosystem). Recombinant CDV was rescued from the cDNA clones as described in Chapter 2. B95a cells were coincubated with 293 cells for 10 days or until the appearance of cytopathic effect (CPE). For confirmation of rescued CDV, RT-PCR was performed using primers for N and P genes (Chapter. 2) and the obtained PCR fragments were sequenced.

Virus characterization

Multistep growth kinetics of the viruses were analyzed after the inoculation of the viruses to B95a cells at an MOI of 0.01 in 12-well plates. Virus was adsorbed to the cells for 1-2 hr and inoculum was removed. Then 1 ml of medium was supplied into each well. The cells were harvested at various times after the inoculation. Virus induced CPE was visualized at 48 hr post infection (hpi). At every 24 hr between 0 to 144 hpi, the cells with medium were harvested and frozen at -80°C. The suspension was frozen and thawed three times and the released virus was determined by endpoint dilution assays to calculate 50% tissue culture infectious dose ($TCID_{50}$) values as standard method.

Preparation of recombinant leishmania antigens and their polyclonal antibodies

TSA- and LmSTI1-cDNA were ligated to E.coli protein expression vector pET32a (Novagen), which recombinant proteins were expressed as fusion proteins with

histidine-tag. The plasmids were transformed into BL21 competent cells for high-level protein expression and one-litter culture was induced to express TSA and LmSTI1 proteins at mid-log phase of growth (0.2 OD_{600}) by addition of 1mM IPTG. After 3 hr incubation, E. coli was pelleted and washed by PBS (-). The pellet was lysed by lysis buffer (1% Triton X-100, 50mM Tris-HCl, 50mM NaCl, 1mM EDTA and 1mM DTT) and centrifuged at 15,000x g for 30 min to remove insoluble materials. Recombinant TSA was found to be mainly produced in soluble fraction, but recombinant LmSTI1 was in insoluble fraction. The TSA protein containing the N-terminal histidine-tag in the supernatant was affinity purified with Ni-nitrilotriacetic acid (NTA) resin column (Amersham Bioscience) according to the manufacturer's protocols with AKTA prime FPLC (Amersham Bioscience). On the other hand, the insoluble pellet of LmSTI1 was lysed with 6M Guanidine-HCl. The supernatant was separated and applied to NTA resin. The column was washed by 6M Urea and refolded by 50mM Tris-HCl with gradient flow. Following elution, the purification was performed as well as TSA. Each leishmania antigen (100μg) were mixed with RIBI adjuvant (Corixa) and immunized to rabbits twice with one month interval. Sera were collected at 35 days after first immunization. Non-specific antibody in the sera were removed with liver powder for 3 hr at 4°C followed by heating at 55°C for 30 min due to inactivation of containing complements. Reactivities of antibodies were confirmed by ELISA and western blot analysis.

Immunofluorescent assay

B95a cells were grown in 10cm plates and infected with recombinant CDVs or the parental Yanaka strain. After 3 days, the cells were removed by cell-scraper, washed once in PBS (-) and dropped onto the wells of FA plates. The cells were dried up briefly

and fixed in acetone for 30 min. The cells were consecutively processed for 60 min with a solution containing polyclonal anti-TSA or LmSTI1 sera (diluted at 1:200) in PBS (-) and then goat anti-rabbit antibody-fluorescein isothiocyanate (FITC) conjugate in PBS (-) (diluted at 1:500) (CAPPEL). After washing in PBS (-) 3 times, imaging analysis was performed with confocal microscopy, Fluoview FV500 system (Olympus).

ELISA

The production of anti-CDV or anti-leishmania antibodies in dog sera was determined with an enzyme-linked immunosorbent assay (ELISA) [Gemma, 1996a]. When anti-CDV antibodies were checked, the extracts of either the Yanaka strain-infected B95a cells or uninfected B95a cells were spread in a 96-well plate. ELISA was performed with the extract or the purified leishmania antigens. The plates were consecutively incubated with various dilutions of dog sera and sheep anti-dog IgG conjugated with horse radish peroxidase (HRP) (CAPPEL) and then with the ELISA substrate (Bio Rad), and optical density values at 570nm were measured.

Western blot analysis

Infected cells were washed once with PBS and lysed in 1% Triton X-100 in 10mM Tris–HCl (pH7.5), 5mM EDTA, 1mM DTT, 0.25mM PMSF. The concentration of the total protein was measured by BCA (PIERCE). Each sample (20μg/lane) was separated by 10%-SDS-polyacrylamid gel electrophoresis under reducing condition with 2-mercaptoethanol and then blotted onto Immobilon-P nylon membranes (Millipore). After blocked by PBS (-) containing 4% block ace (Yukijirushi) and 0.05% Tween-20, the membranes were incubated in the antibody solution containing 1:500 dilution of

anti-sera immunized by each antigen in PBS for 1 hr at room temperature. After washed, the membranes were incubated with anti-rabbit IgG conjugated with HRP (CAPPEL) and then treated with ECL western blotting detection reagent. The reaction was visualized by LAS1000 (Fuji film).

Northern blot analysis

Total cellular RNA of infected or mock-infected B95a cells was prepared by guanidine thiocyanate method using Isogen. Fifteen μg of total RNA was separated in 1% agarose/formaldehyde gel subsequently transferred to Hybond-N^+ (Amersham Bioscience). The membrane was UV-crosslinked for 3 min, and hybridized with specific probes encoding leishmania antigens or CDV-N genes. For the N gene specific probe, BamHI fragment of the N gene cDNA was used. The other probes were amplified with original primers of ORF region by PCR, and the PCR products were purified by QIAEXII (QIAGEN). The probes were labeled using Alk Phos labeling kit (Amersham Bioscience) reacted with ^{32}P–dCTP. The hybridization was carried out according to manufacturer's instruction. The membrane were exposed to IP plate and the detection was performed using BAS2000 (Fuji film).

Vaccination and challenge experiments

Eight 5-weeks-old female beagles (Nihon Nosan), which do not have any antibodies against CDV in the sera were used, ranging the weight of 0.7 to 0.8 kg. Dogs were immunized with TSA-CDV (n=4), LmSTI1-CDV (n=4), their mixture including LACK-CDV (n=4), or the parental Yanaka strain (n=4) at day 0 and 14. The inoculum was 500μl at dose of $10^{4.5}$ $TCID_{50}$ per ml, subcutaneously. Body temperature, body

weight and clinical signs were checked daily for 21 days. Leukocyte count and antibodies against CDV were also checked at 0, 28 and 35 days after the first immunization. Infective-promastigotes of *L. major* were inoculated at $5X10^7$ parasites per spot intradermally into ears at 42 days after the first immunization. Every week after the challenge, the sizes of the nodules on the ears were determined by its area (mm^2). Blood samples for WBC count and sera were collected every 7 week. At 84 days after the challenge, the dogs were euthanized and the tissues were collected. Parasites in the tissues were observed by histological examination [Terabe, 2000]. Any animal experiments author carried out were performed in accordance with the Manual for Animal use and care of the University of Tokyo.

Determination of the IgG2/IgG1 against leishmania in the dog sera

The IgG1 and IgG2 levels against *leishmania* were measured to reveal the Th1 or Th2 immunodominant type against leishmania. Leishmania promastigotes were lysed by Salch buffer (0.5% Triton X-100, 0.5% DOC, 5mM NaCl and 25mM Tris-HCl) and 10ug antigens were coated on the ELISA plate for overnight at 4℃. Dogs antibodies were treated with liver powder and heat inactivated the serum complement. After 2 hr 4% block Ace/PBS (-), serially diluted serum with carbonate buffer were applied on antigen coated ELISA plate and incubated for 1 hr at room temperature. After three times brief wash in 0.05% Tween-20/PBS (-), anti dog IgG1 or IgG2-HRP conjugate were applied and incubated 1 hr at room temperature. After several washing, MTB solutions (Bio Rad) were applied to ELISA plate and incubated for 30 min. The ELISA plates were measured by plate reader (Bio Rad) at 570nm.

RESULTS

Rescue of recombinant CDVs

The CDV full genome plasmid (pCDV) for reverse genetics system [Fujita, 2002] was based on a field isolate of the Yanaka strain, which we separated previously [Gemma, 1996b]. Dogs infected with the Yanaka strain had no observable CD symptom and showed protective effect against the challenge with virulent CDV [Takenaka, in preparation]. Therefore, the Yanaka strain was considered to be avirulent and a good candidate for the CDV vaccine strain. As *Leishmania* antigens for recombinant CDVs, TSA (Homologue of eukaryotic thiol-specific antioxidant), LmSTI1 (Leishmania stress-inducible protein 1) and LACK (Leishmania homologue of receptor for activated protein kinase C) were selected. All the antigens are expressed in the internal leishmania and immunization of the antigens or the cDNA induced cellular immunity against leishmania infection in mice [Webb, 1996; Webb, 1998; Mougneau, 1995]. TSA and LmSTI1 genes were obtained from *L. major* and coded for entirely 200 amino acid (aa) and 537 aa, respectively. The sequences were identical to the previous reports [Webb, 1996; Webb, 1998]. LACK gene from *L. donovani* coded for the entirely 312 amino acids, which possessed only three amino acid differences from those of *L. major* [Okuno, 2002]. Recombinant LACK-CDV was successfully established previously [Miura, 2002]. Each entire open reading frame (ORF) was attached with a set of transcriptional end signal and start signal at the upstream of the ORF (Fig. 1). The plasmids were applied for our standard CDV rescue system using 293 cells and MVA-T7 [Fujita, 2002]. At three days after cocultured with B95a cells, a typical cytopathic effect (CPE) was observed in all three cultures for rescuing recombinant CDVs (Fig. 2A). The rescued viruses were designated as TSA-CDV and LmSTI1, and were confirmed by RT-PCR using

each set of specific primers, which encoded the N gene and each antigen gene. The amplified DNA fragments were identical to each antigen gene sequence.

Viral growth kinetics and expression of antigens in the recombinant CDVs

Author examined the CPE formation and virus growth kinetics after B95a cells were infected with the recombinant CDVs or its parental Yanaka strain (Fig. 2A). The size of CPE formed by the recombinant CDVs was comparatively smaller to the parental strain (Fig 2A). The rescued CDVs grew slower than the parental Yanaka strain but reached to equivalent maximum titers. The growth kinetics showed no significant difference (Fig. 2B). On the other recombinant CDVs that we previously rescued with insert of EGFP (0.8 kbp) or luciferase (1.6 kbp), there were also no observable differences in sizes and growth kinetics compared with the Yanaka strain [Fujita, 2002]. Since gene length of all the insertion was within 1.6 kb, the inspection may not be affected on their replication. Northern blot analysis showed that each mRNA of gene was detected as both monocistronic- and bicistronic mRNA (Fig. 3). The monocistronic mRNA can translate the leishmania antigen proteins, whereas the bicistronic mRNA can produce only N protein but not the antigen protein. To investigate the expression of the antigens and to measure the production of antibodies by ELISA, recombinant TSA and LmSTI1 proteins were produced by bacteria and successfully purified by Ni-NTA column (Fig. 4A, B). The specific antibodies were produced by rabbit immunized with the recombinant antigens. Using the antibodies, B95a cells infected with the recombinant CDVs were analyzed with immunofluorecence (Fig. 5) and western blot (Fig. 6). In immunofluorescent analyses, these antigens were mainly expressed in cytoplasm in the infected cells. The molecular weights of these proteins were identical to expected

molecular weights, 22 and 62 kDa. These results demonstrated that both of the recombinant CDVs produced the antigens well in infected cells.

Immunization of recombinant CDVs

To evaluate the safety and efficacy of the recombinant CDVs, the author carried out animal experiments using dogs. The 5-weeks-old beagles, which were negative for the antibody against CDVs were used. Body weight was measured daily and all dogs either immunized or mock-treated showed an increase of body weight until 21 days after the first immunization (Fig.7A-C). The 6 dogs immunized with the recombinant CDVs showed no clinical signs of CD, such as pyrexia or leukopenia. All dogs were observed for 6 weeks, but no clinical signs were observed. Thus, the recombinant CDVs are considered to be safe for immunization. The antibody responses were analyzed by using ELISA. CDV antibodies were significantly detected in all dogs from 21 days, indicating that the recombinant CDVs promoted host immunity against CDV, whereas the antibody against TSA or LmSTI1 antigens was produced at very low or undetectable levels until 50 days post injection. These results are almost identical to those of the experiment of immunization with the parental Yanaka strain [Takenaka in preparation] and LACK-CDV [Miura, 2002].

Protection against *L. major* challenge

The efficacy of immunization with TSA-CDV and LmSTI1-CDV and three mixture including LACK-CDV against *leishmania* were evaluated. *L. major* intradermally inoculated in dogs usually proliferates at the site of inoculation and forms a nodule in the skin lesion. The parasites' growth was evaluated as the nodule sizes. In this experiment,

high dose of *L. major* was inoculated into ears and noses. The antibody against *Leishmania* was measured by using ELISA up to 5 weeks, and at a week after the challenge all dogs showed high titers of the antibody (data not shown). The diameter of nodule sizes on ears was measured for 10 weeks (Fig. 8). The nodules in the unimmunized, CDV Yanaka-immunized, TSA-CDV-immunized and LmSTI1-immunized dogs first appeared in the 2nd week and the ulcers appeared in the 3rd week. The nodules enlarged rapidly, reached at a maximum size between 3rd and 6th week and then regressed. On the other hand, the nodules in the triple mixture immunized dogs were smaller than those in the unimmunized dogs and ulcerated up to 6 weeks after the challenge. In addition, the nodules of the triple mixture immunized dogs reduced quite rapidly (Fig. 8E). The nodules on noses were observed similar as on ears. These results showed that the triple mixture immunization was most effectively suppressed the proliferation of *Leishmania* at all stage. All dogs were euthanized at the 10-12 weeks after the challenge. The tissues of ears from control, TSA-CDV, LmSTI1-CDV and the triple mixture immunized dogs were collected and histochemically analyzed. *Leishmania* could not be detected in all of the tissue sections (data not shown).

Determination of the IgG2/IgG1 against leishmania in the dog sera

Since Th1/Th2 ratio is considered to be estimated by IgG2/IgG1 ratio, the author analyzed the ratio. All dogs except LmSTI1-CDV immunized ones showed high IgG2/IgG1 ratio at 3 weeks after the leishmania challenge (Fig. 9). High IgG2/IgG1 shows the Th1 dominant immune reaction, and low IgG2/IgG1 shows Th2 dominant immune reaction. Specific effect of the increase of the Th1 dominant immune reaction, caused by the immunization of recombinant CDVs were not observed by this analysis.

LmSTI1 immunized dog showed high response in both IgG1 and IgG2, which meant LmSTI1 could induce both Th1 and Th2 immunoreactions. This result was consistent to the previous LmSTI1-DNA vaccine study.

DISCUSSION

Author displayed here recombinant CDVs for use as effective polyvalent vaccine candidates against CDV and *Leishmania* infections. The results showed that CDV was a safe and effective viral vector and a combination with TSA-CDV, LmSTI1-CDV and LACK-CDV could effectively suppress the proliferation of *L. major* in all stage of infection.

The susceptive mice (BALB/c) showed the dominance of IL-4-driven Th2 response, whereas resistant mice (C57BL/6) showed IL-12 driven, IFN-γ dominant Th1 response that promotes healing and parasite clearance [Sacks, 2002]. Thus, the *L. major* infection model in mice indicated that resistance to *L. major* infection was correlated with the Th1 response. Most of candidates of the *leishmania* antigens for leishmania vaccination, including LACK, TSA and LmSTI1, was found and cloned by the specific mice model [Mougneau, 1995; Webb, 1998; Campos-Neto, 2002]. As recombinant proteins, LACK and TSA have produced at least partial protection against *L. major* in BALB/c mice [Mougneau, 1995; Webb, 1998] and LmSTI1 DNA was shown to induce high level of IFN- γ and IL-4 in lymph node cells of infected mice [Campos-Neto, 2002]. These antigens show different responses in immunized mice. The different effects of the recombinant CDVs observed in the present study are considered to be dependent on the properties of antigens and the immune responses of outbred dogs.

All the leishmania antigens in this study are usually expressed and localized in the internal leishmania body. In construction of recombinant CDVs, the antigen genes have no genetic modification for secretion and location on the cell surface, and the antigens were stored in B95a cells infected with the recombinant CDVs. This probably caused low or undetectable level of specific antibody production in sera of the infected

dogs. Measles virus (MV) and Rinderpest virus (RPV) N protein can induce cytotoxic T lymphocyte [Fooks, 1995; Ohishi, 1999]. Thus, internal antigens in recombinant CDVs also have an ability to activate cell-mediated immunity.

In this study, TSA-CDV could not show efficient protection although previous study supplied good protection against *L. major* challenge used as DNA vaccine in mice [Campos-Neto, 2002]. This result might be influenced by the high challenge dose used in the present study, which was 250 times higher than the mouse experiment and over 10^5 times higher than natural infection via sandy fly bite. In case of LmSTI1, the result of protection is similar to DNA vaccine experiment, which LmSTI1 induced both Th2 and Th1 induction at the same time [Campos-Neto, 2002]. The dogs immunized with CDV-Yanaka alone showed the reduction of nodule size in the early stage similar to that in dogs immunized with TSA or LmSTI1 immunization. The mechanisms of the effects were unknown; however, activated immune state caused by CDV immunization may induce rapid reaction against leishmania infection.

TSA-, LmSTI1- and LACK-CDV mixed immunization showed a most effective result for *L. major* challenge than TSA- or LmSTI1-CDV. From the previous report of TSA- or LmSTI1-DNA vaccine in BALB/c mice [Campos-Neto, 2002], most protective effect was expected by TSA-CDV immunization. However it did not show effectiveness compared with LmSTI1-CDV or CDV-Yanaka immunization. Additional LACK-CDV immunization accelerated protective immunity against *L. major* challenge in all the stage of leishmaniasis. LACK had been used as a new leishmania vaccine candidate and mostly showed good protection against *L. major* [Mougneau, 1995; Gurunathan, 1998; Stobie, 2000]. In previous report, LACK was presented on the surface of macrophage by MHC class II and induced CD4+T cell activation [Launois, 1997]. Immunization of

recombinant LACK protein with IL-12 confers high level of protection in BALB/c mice against *L. major* challenge [Stobie, 2000; Mougneau, 1995]. LACK-DNA and Vaccinia LACK prime-boost vaccine also showed protection against *L. infantum* in Beagle dogs with both IFN-γ and IL-4 induction [Ramiro, 2003]. Although the effect of elevated Th1 reaction caused by the recombinant CDVs immunization in the analysis of IgG2/IgG1 ratio, the recombinant CDVs induced significant effects of suppression of *Leishmania* growth, which effect was much higher than the previous reports using protein or DNA vaccination [Webb, 1996; Coler, 2002; Campos-Neto, 2002]. Thus the recombinant CDVs are one of most effective vaccine candidate against leishmania infection.

Many viral rescue systems for the order *Mononegavirales* were reported. Most of the rescued viruses including other CDV, MV and RPV were based on their vaccine strains. On the other hand, the genome sequence of our rescued CDV was identical to the Yanaka strain [Fujita. 2002], a recent field strain which we isolated [Gemma, 1996b]. The vaccination with the recent isolate is expected to induce more effective immunity than with the vaccine strains which were developed at least 40 years before [Bolt, 1997]. Previous studies confirmed that the parental Yanaka strain itself is safe in dogs and that dogs vaccinated with the Yanaka strain completely protected against virulent CDV, Snyder Hill strain challenge [Tekenaka in preparation; Miura, 2002]. In this study, author succeeded to establish recombinant leishmania vaccines for dogs using the CDV Yanaka strain, and this study showed as powerful tools for the prevention of leishmaniasis epidemics by disruption of infection root in dogs. The recombinant CDVs based on the Yanaka strain is considered to be superior to conventional vaccine strains as a polyvalent vaccine vector.

FIGURE LEGENDS

Fig 1: Schematic diagram of rescuing recombinant TSA-CDV and LmSTI1. Each cloned *leishmania* antigen cDNA was added at FseI site. Each fragment was digested by FseI enzyme and ligated between N and P genes in pCDV full genome plasmid. The prepared plasmids were transfected into 293 cells with supporting N, P and L plasmids. At 3rd day, B95a cells were cocultured with the 293 cell and CPE were observed at 5th day, indicating that recombinant virus rescue was succeeded.

Fig 2: Comparison of CPE and growth kinetics of the rescued viruses. (A) There were no significant differences of CPE between TSA-CDV or LmSTI1-CDV and the parental CDV-Yanaka. (B) The rescued CDVs grew slower than the parental CDV-Yanaka, but reached to equivalent maximum titers. The differences from the parental Yanaka strain to recombinant CDVs were not significant.

Fig 3: Northern blot analysis of inserted *leishmania* antigen expression of the rescued CDVs. Total RNA from recombinant CDVs infected or mock-infected B95a cells were analyzed by northern blot with probes encoding *leishmania* antigen. Each antigen mRNA was detected as both monocistronic (arrows at lower) and bicistronic mRNA (arrows at upper).

Fig 4: Recombinant proteins TSA and LmSTI1, fused with tag protein (histidine-tag and TRX, thioredoxin) were expressed in E.coli and analyzed by coomassie staining (A) and western blot (B). The cultures were separated to LB medium fraction, periplasm, soluble fraction and insoluble fraction. After SDS-PAGE, one gel was visualized by coomassie staining. Arrows indicate the recombinant TSA and LmSTI1. In western blot analysis, since the tag protein is estimated at 23kDa of molecular weight, LmSTI1 and TSA were shown as fusion protein of 85kDa and 45kDa, respectively. t: only tag protein, T: TSA fusion protein, L: LmSTI1 fusion protein.

Fig 5: Immunofluorescent assay of B95a cells infected with the recombinant CDVs. B95a cells infected with TSA-, LmSTI1-CDV or parental CDV-Yanaka were fixed and reacted with anti-TSA or LmSTI1 polyclonal antibodies (1:200) diluted in PBS and then with goat anti-rabbit antibody-fluorescein isothiocyanate (FITC) conjugate in PBS (1:500). The cells were visualized by confocal microscopy. All of integrated *leishmania*

antigens were well detected in the cytoplasm. (200x magnification)

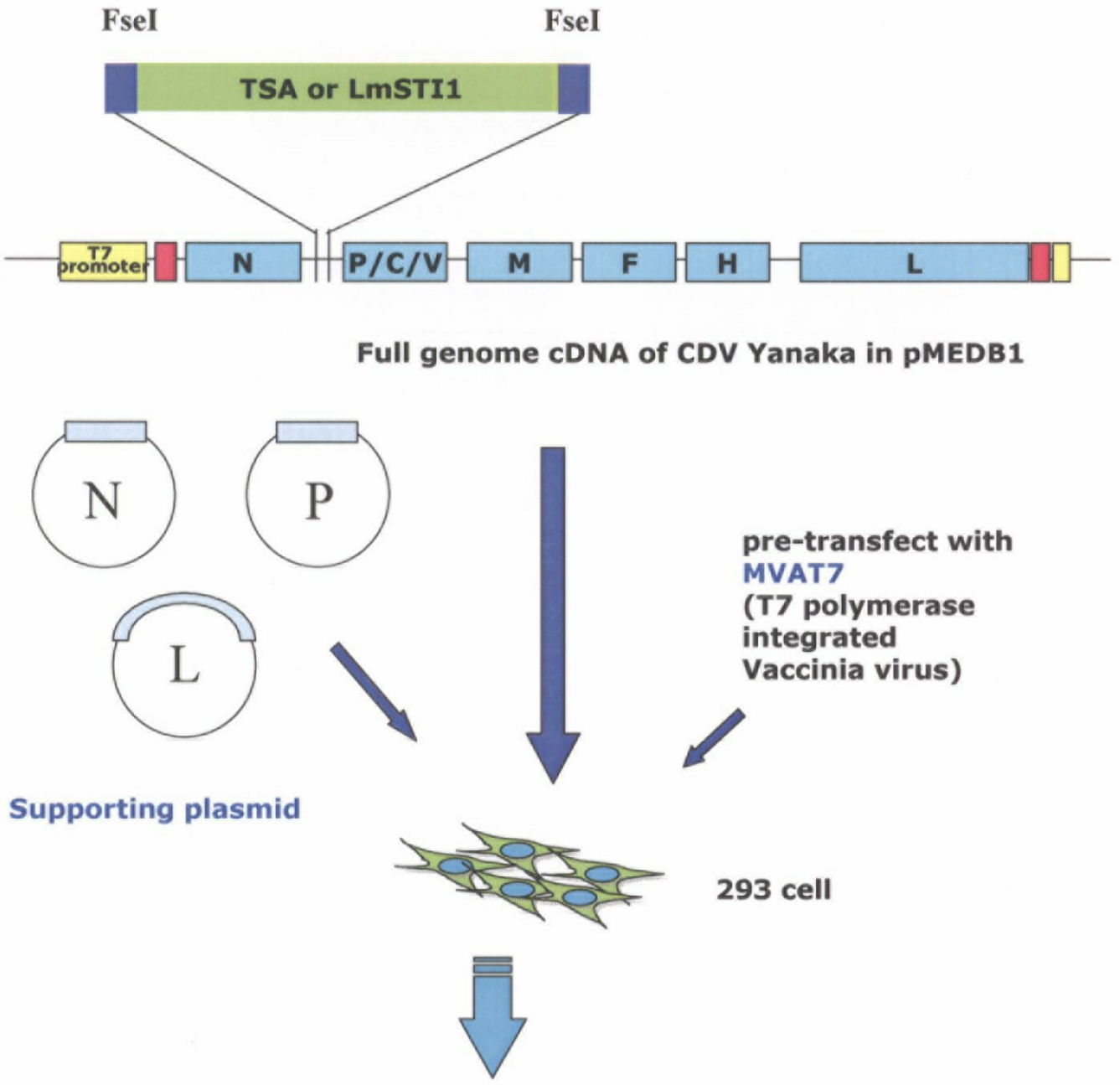
Fig 6: Western blot analysis of B95a cells infected with the TSA-CDV and LmSTI1-CDV. The cells were lysed and applied to SDS-PAGE. Transferred membrane was blocked and reacted with anti-TSA or LmSTI1 polyclonal antibody (1:500). Membrane was post stained by anti rabbit HRP conjugate as second antibody colored with chemiluminesence, and then detected by LAS1000. Expected TSA (22.1kDa, lower band) or LmSTI1 (62.1kDa, upper band) bands were detected.

Fig 7: A: Body weights (A) body temperatures (B) and leukocyte count (C) of TSA-CDV, LmSTI1-CDV, mixture (TSA-CDV, LmSTI1CDV and LACK-CDV), parental CDV-Yanaka immunized dogs and non-immunized control dogs after immunization. Arrows show the day of vaccination and triangles indicate the day of *leishmania* challenge. (A) All dogs showed no body weight loss either after CDV immunization or after *leishmania* challenge. (B) After CDV immunization, all dogs showed slight increased of body temperature but not pyrexia. After *leishmania* challenge, all dogs showed no pyrexia. (C) All dogs showed unstable leukocyte count but neither leukocytosis nor leukopenia.

Fig 8: Nodule sizes of dogs after the leishmania challenge. A-E indicated each dog. (A) The nodule sizes of control dogs began to increase at 2^{nd} week, reached peak at 3^{rd} week. They kept the size until 5^{th} week and then regressed. (B) The nodule sizes of CDV-Yanaka immunized dogs began to increase at 2^{nd} week, reached peak at 4^{th} or 5^{th} week, and then regressed. (C and D) The nodule sizes of TSA-CDV or LmSTI1-CDV immunized dogs began to increase at 2^{nd} week, reached peak at 4^{th} or 5^{th} week, and then regressed. The sizes of them especially LmSTI1-CDV immunized dogs were smaller and the regression speeds were faster than control dogs. (E) Nodule sizes of mixture (TSA-CDV, LmSTI1CDV LACK-CDV) immunized dogs began to increase at 2^{nd} week, reached peak at 5^{th} week, and then regressed. The sizes were significantly smaller than control dogs during all stages of infection and then regressed very rapidly. Then the nodules were disappeared by 8^{th} week.

Fig 9: IgG2/IgG1 ratios of non-, CDV-Yanaka, TSA, LmSTI1 or mixture (TSA-CDV, LmSTI1CDV LACK-CDV) immunized dogs after the leishmania challenge. LmSTI1-CDV immunized dogs (G and H) showed lower IgG2/IgG1 ratio than the other dogs. Triangle indicates the day of leishmania challenge.

Fig. 1


FseI
FseI
TSA or LmSTI1
T7 promoter
N
P/C/V
M
F
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Full genome cDNA of CDV Yanaka in pMEDB1
N
P
L
pre-transfect with
MVAT7
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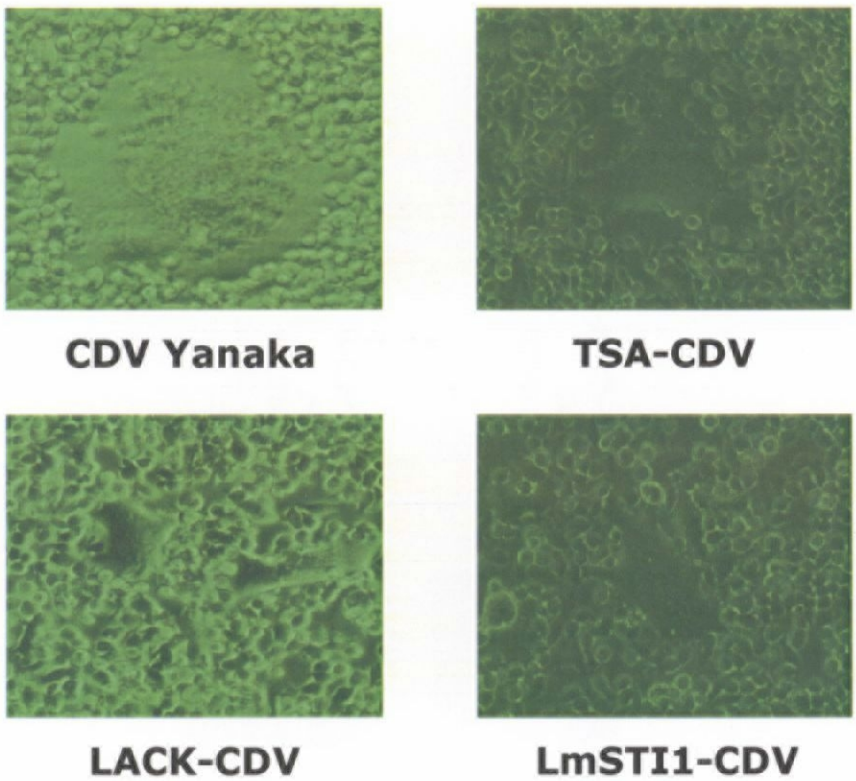
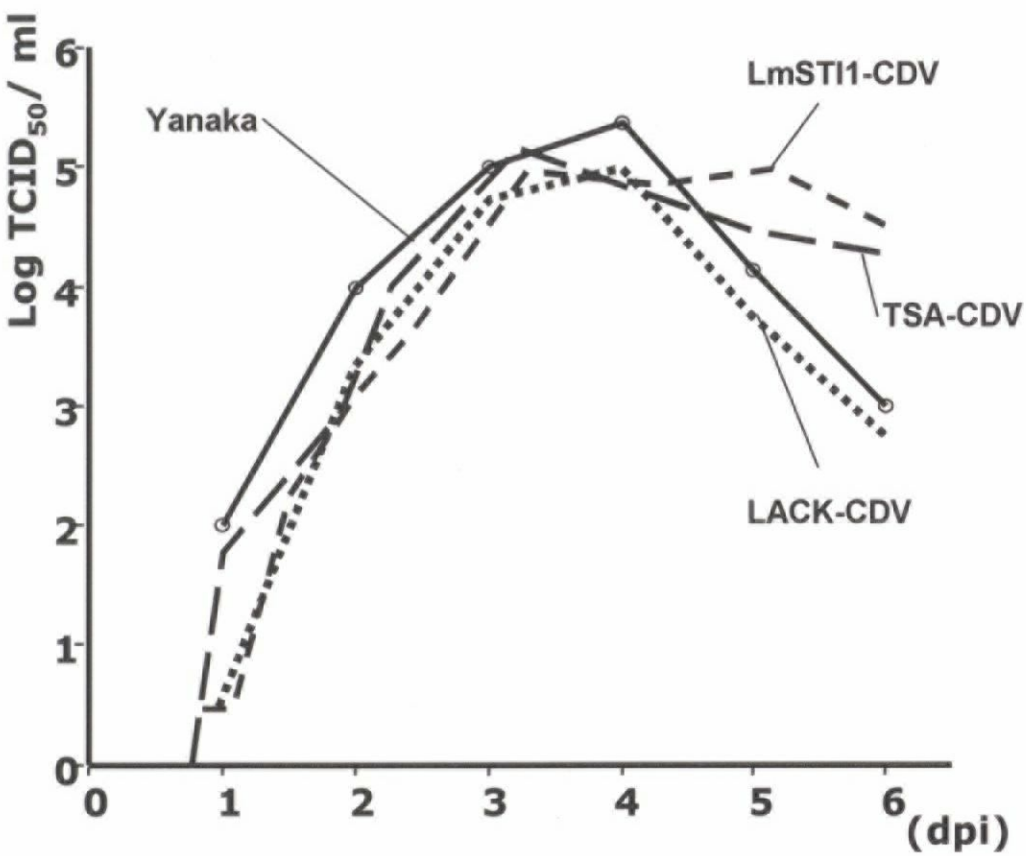
integrated
Vaccinia virus)
Supporting plasmid
293 cell
Add B95a cell and identify the CPE
Then the recombinant viruses were rescued

Fig. 2

(A)


CDV Yanaka
TSA-CDV
LACK-CDV
LmSTI1-CDV

(B)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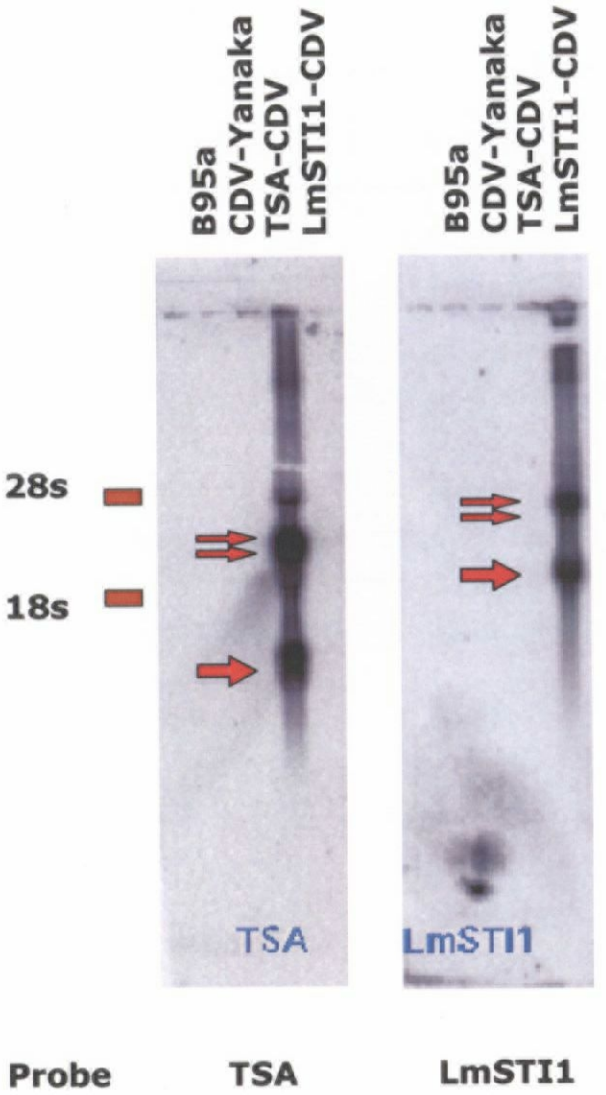
1
2
3
4
5
6
Yanaka
LmSTI1-CDV
TSA-CDV
LACK-CDV
0
1
2
3
4
5
6
(dpi)

Fig. 3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TSA-CDV
LmSTI1-CDV
B95a
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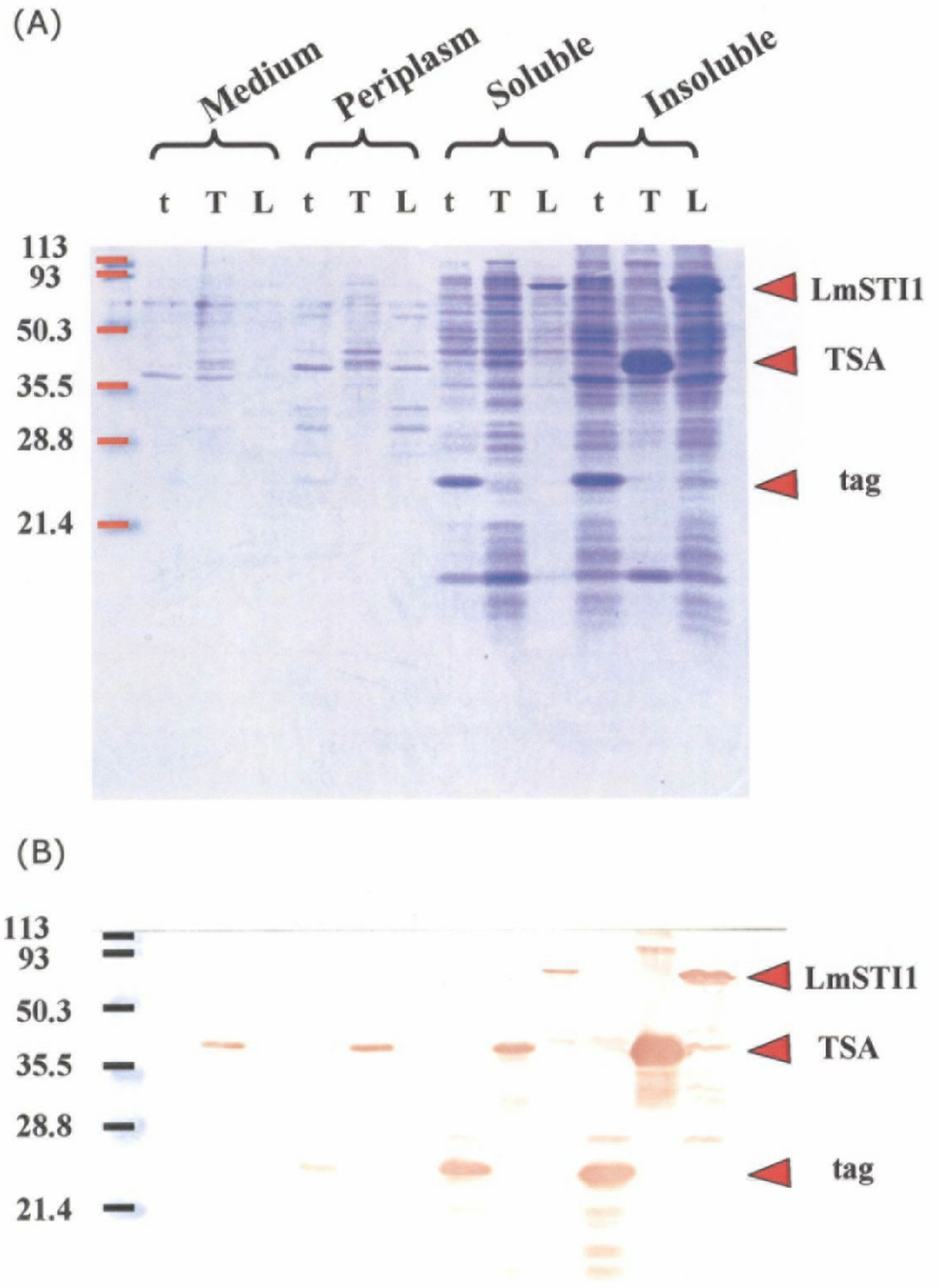
18s
TSA
LmSTI1
Probe
TSA
LmSTI1

Fig. 4


(A)
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Soluble
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93
50.3
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LmSTI1
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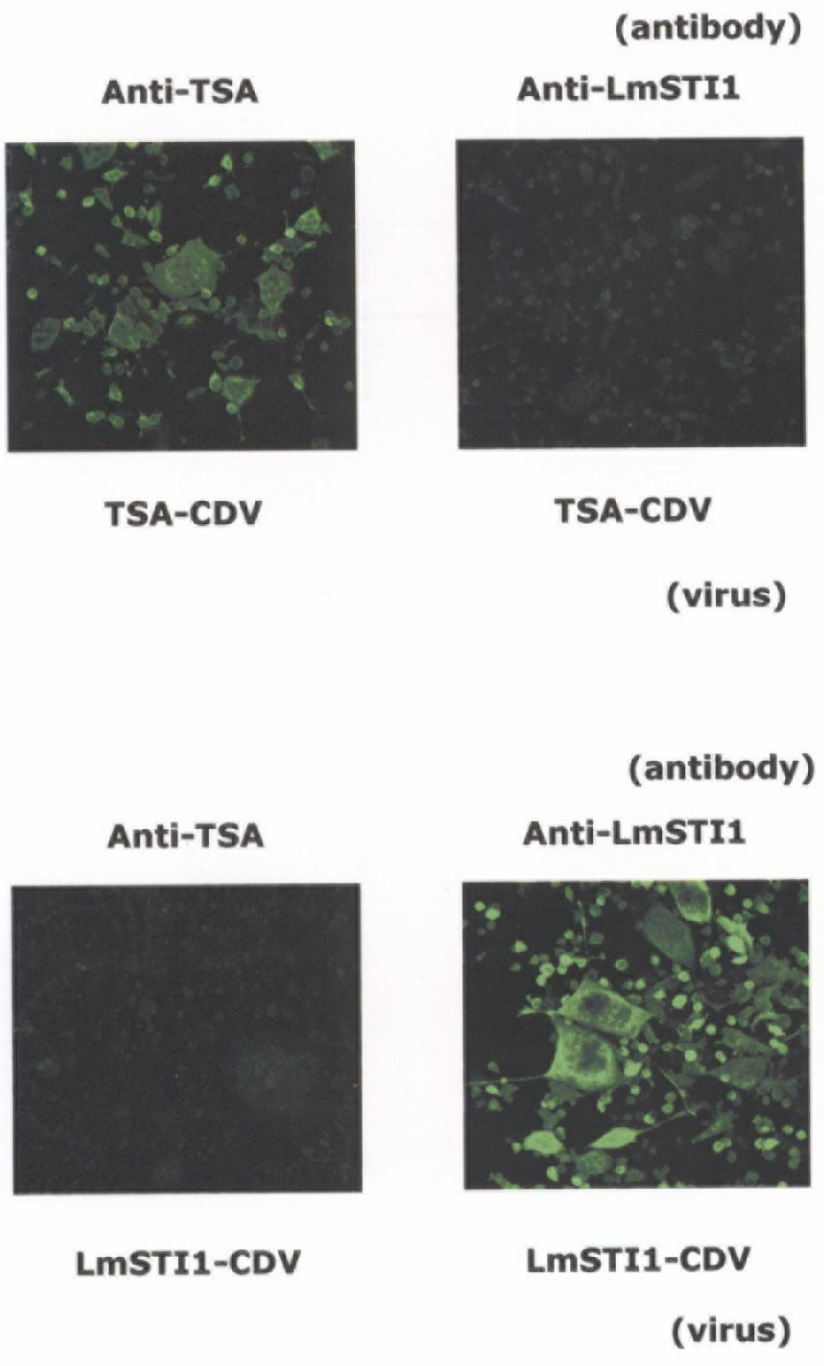
TSA
tag

Fig. 5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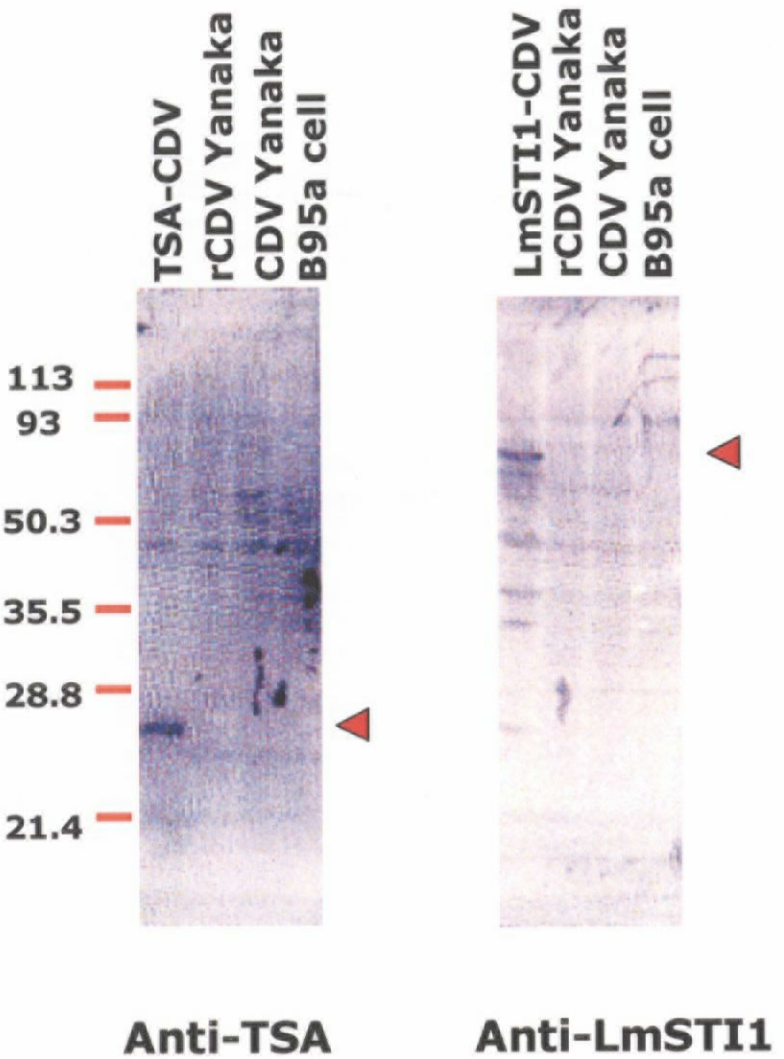
LmSTI1-CDV
(virus)

Fig. 6


TSA-CDV
rCDV Yanaka
CDV Yanaka
B95a cell
LmSTI1-CDV
rCDV Yanaka
CDV Yanaka
B95a cell
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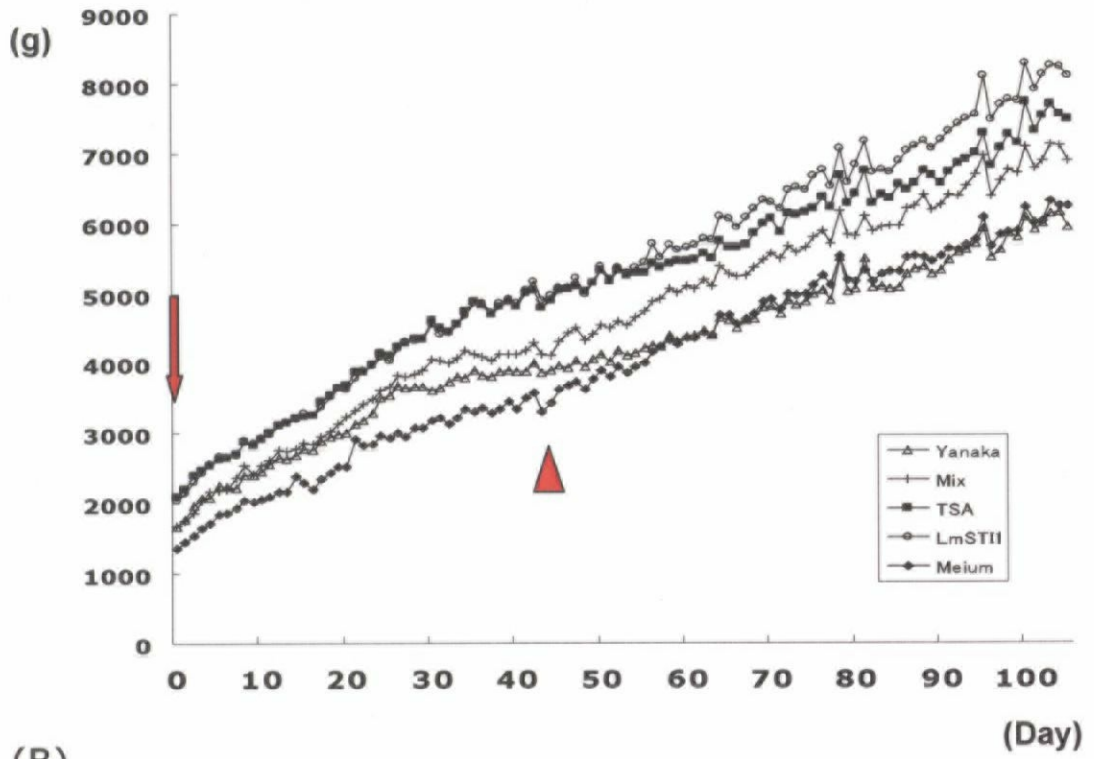
93
50.3
35.5
28.8
21.4
Anti-TSA
Anti-LmSTI1

Fig. 7

(A)


(g)
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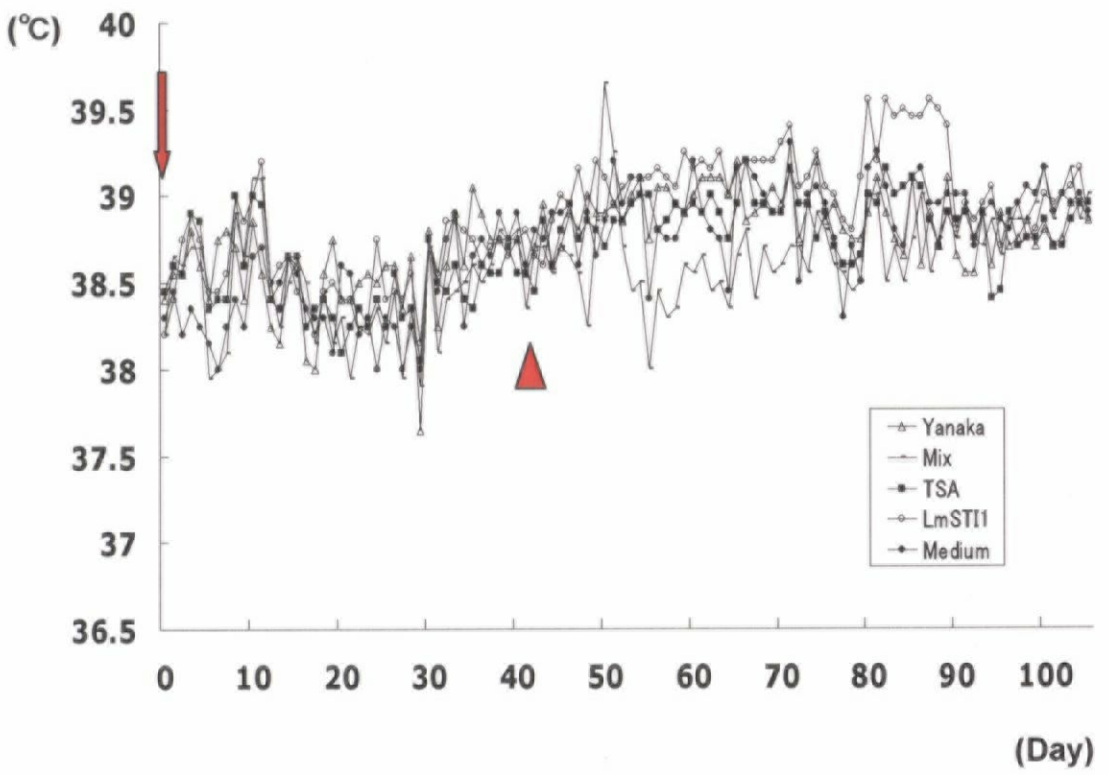
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
100
(Day)
Yanaka
Mix
TSA
LmSTI1
Meium

(B)


(℃)
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39
38.5
38
37.5
37
36.5
0
10
20
30
40
50
60
70
80
90
100
(Day)
Yanaka
Mix
TSA
LmSTI1
Medium

Fig. 7

(C)

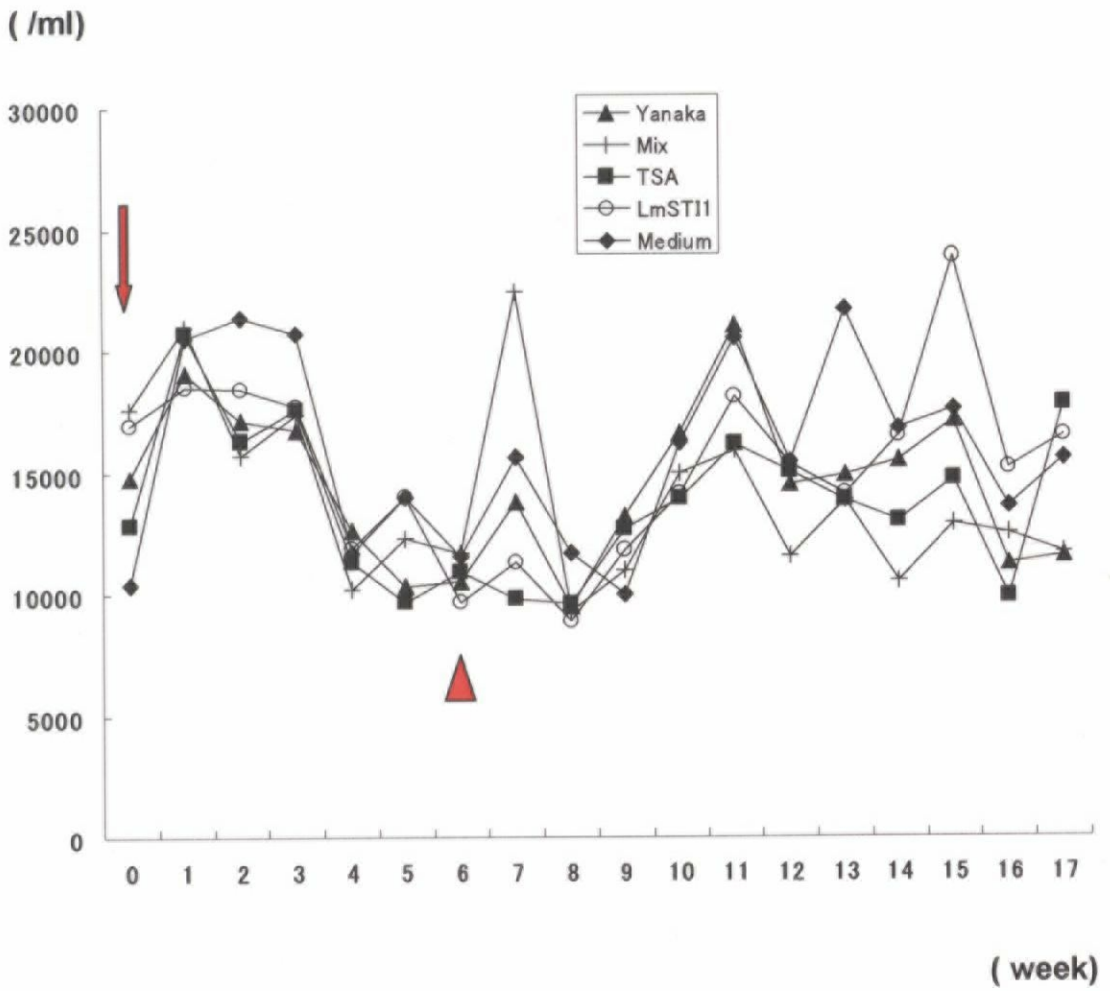
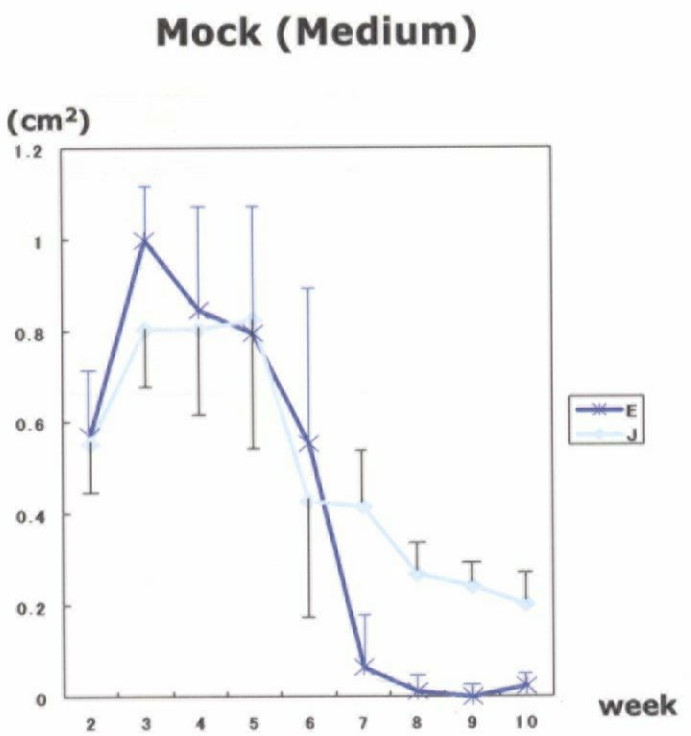

(/ml)
30000
25000
20000
15000
10000
5000
0
Yanaka
Mix
TSA
LmSTI1
Medium
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
(week)

Fig. 8

(A)


Mock (Medium)
(cm2)
1.2
1
0.8
0.6
0.4
0.2
0
2
3
4
5
6
7
8
9
10
week
E
J

(B)

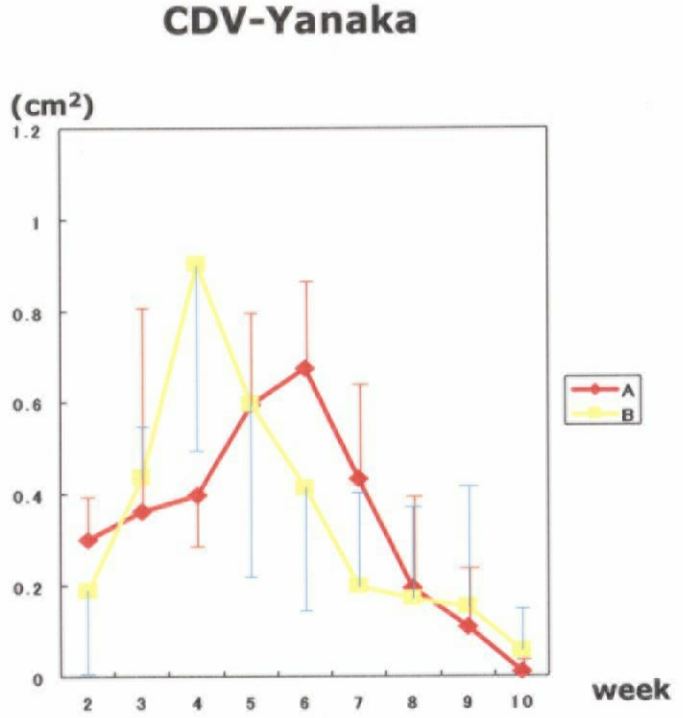
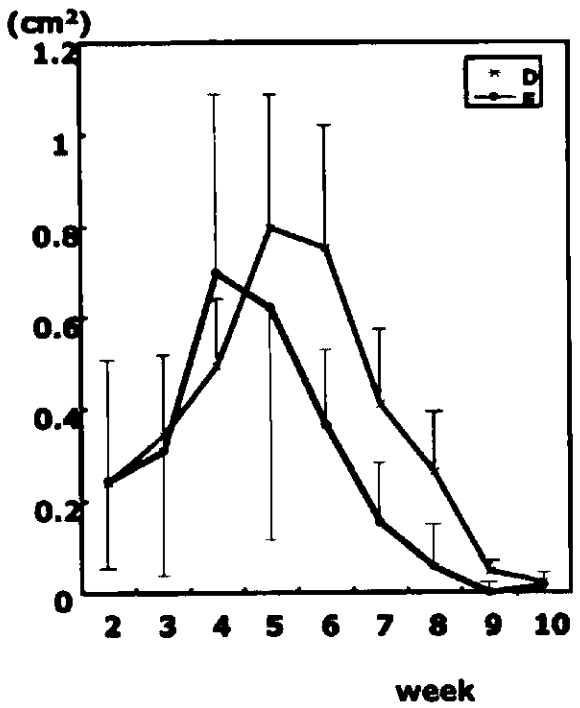
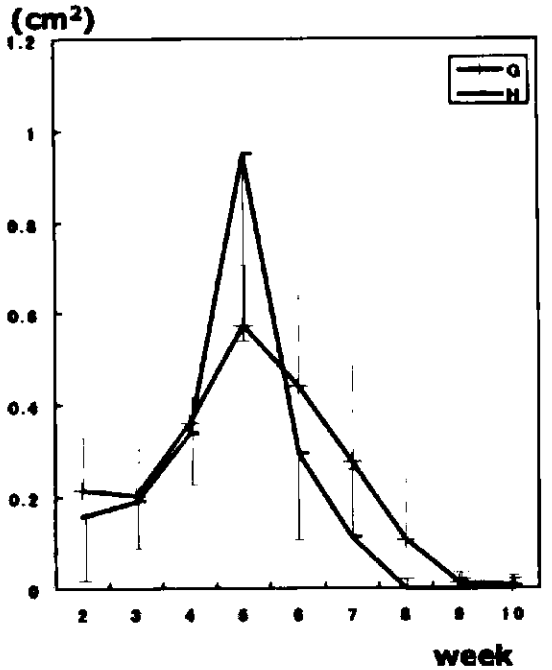

CDV-Yanaka
(cm2)
1.2
1
0.8
0.6
0.4
0.2
0
2
3
4
5
6
7
8
9
10
week
A
B

Fig. 8

(C)


TSA-CDV
(cm²)
1.2
1
0.8
0.6
0.4
0.2
0
2 3 4 5 6 7 8 9 10
week

(D)


LmSTI1-CDV
(cm²)
week

(E)

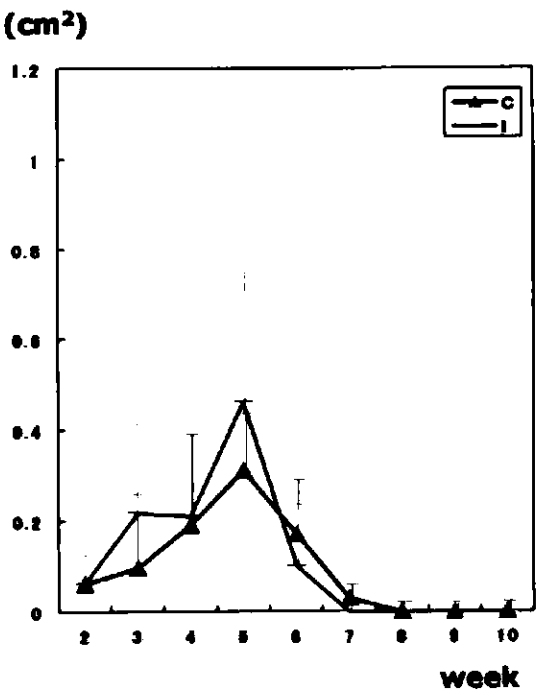
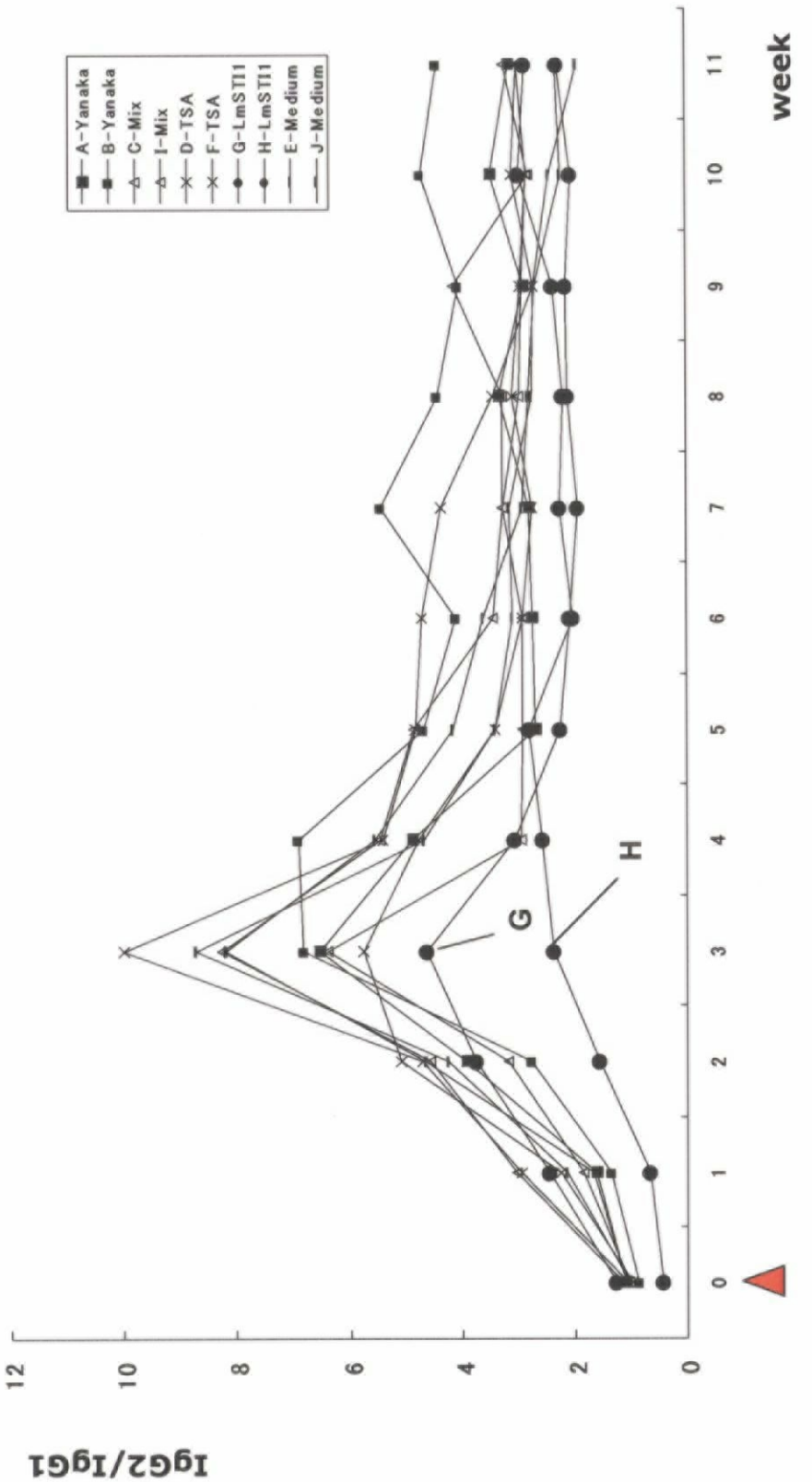

Triple-CDV
(cm²)
week

Fig. 9


A-Yanaka
B-Yanaka
C-Mix
I-Mix
D-TSA
F-TSA
G-LmSTI1
H-LmSTI1
E-Medium
J-Medium
G
H
week
IgG2/IgG1
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
9
10
11