CHAPTER 4

Epitope Mapping of Canine Distemper Virus Phosphoprotein by Monoclonal Antibodies

SUMMARY

Canine distemper virus (CDV) is a member of genus *Morbillivirus* with Measles virus (MV) and Rinderpest virus (RPV), and the virus possesses six structural proteins. Phosphoprotein (P) gene encodes three different proteins, P, V and C, and is related to virus gene transcription. In Chapter 4, author produced monoclonal antibodies (MAbs) against CDV P protein in order to analyze the functions of P protein. P gene of the CDV Yanaka strain, not V nor C region, was cloned into pQE E.coli expression vector, and P protein was produced in mass culture and purified with Ni affinity column. By immunizing the expressed protein into BALB/c mice, author obtained 7 monoclonal antibodies (13Ea, 52G, 36Ba, 33Ba, 36E 42Ba, and 99Bb). Competitive binding assay revealed that they recognizing two antigenic sites of P protein. The first site was interestingly conserved in other recent field isolates and in RPVs, but not in old type CDVs nor in MVs. The second site was appeared specifically only for the Yanaka strain.

INTRODUCTION

In negative-stranded RNA viruses, phospho (P) protein has been shown to have multiple functions. Transcription initiates at the 3′ end of the genome RNA, giving the sequential synthesis of (+) strand leader (le+) RNA and then the N, P/C/V, M, F, H, and L mRNAs [Mellon, 1978; Lamb and Kolakofsky, 2001]. During genome replication, synthesis of viral RNA and encapsidation by N protein are concomitant. N protein has the capacity to self-assemble on cellular RNA to form nucleocapsid-like particle in the absence of viral RNA and viral proteins [Fooks, 1993]. Association of P protein with the soluble, monomeric form of N (N^0) protein prevents it from binding to cellular RNA [Curran, 1995; Huber, 1991]. The N^0-P complex is the substrate used by the polymerase to initiate encapsidation of genomic RNA [Spehner, 1997]. N forms complexes with P and with the P-L complex during transcription and also during replication, in its self-assembled form [Horikami, 1992]. Structural studies show that SeV P protein is a tetramer and the oligomerization domain has a coiled coil structure which extends to aa 433 aa through part of the L protein binding domain [Tarbouriech, 2000a; Tarbouriech, 2000b]. MV P protein is also an oligomer, although the nature of the complex has not been determined [Curran, 1995; Harty and Palese; 1995; Liston, 1995]. RPV P protein is reported to have a key role in the replication of the virus in the cross species infection and pathogenicity using animal model of rabbit and recombinant viruses based on the RPV-L strain [Yoneda, 2004].

All paramyxovirus P proteins are likely to be oligomeric, containing heptads sequence repeats in the central region (approximately 340-410aa) of the molecule, characteristic of helical coiled coils [Curran, 1995]. The structure of this region of the SeV P protein has determined and is a tetrameric coiled [Tarbouriech, 2000]. The

extreme of C terminus of P is critical for nucleocapsid binding, as short SeV eliminate or dramatically decrease N-RNA. The final 50 amino acids of P protein are predicted to form a trihelical structural motif in many paramyxoviral P proteins, including MV [Curran, 1995]. Recent crystal structure of this domain from MV P protein shows it to be a compact bundle of three alpha-helices [Johansson, 2003]. For MV, the C-terminal region of P protein including the putative coiled coil and down stream sequences, can bind to the N protein [Huber, 1991; Liston, 1995; De, 2000; Longhi, 2003].

In Chapter 4, to understand the multifunction of P protein, author established and characterized monoclonal antibodies (MAbs) against CDV P protein of the CDV Yanaka strain.

MATERIALS AND METHODS

Cells and viruses

B95a cells and Vero cells were maintained in RPMI1640 containing 5% FCS and in DEME containing 10% FCS respectively. The Onderstepoort (vaccine strain), Snyder Hill (old virulent strain), Haku93 and Hake00 [Japanese field isolates from masked palm civets, Hirama 2004] of CDV strains, the RBOK (vaccine strain) and L (lapinized strain) of RPV strains, and the Edmonston (vaccine strain) and HL (virulent strain) of MV strains were used. The Onderstepoort, Snyder Hill, RBOK, and Edmonston strains were grown in Vero cells and the others in B95a cells.

Production of recombinant CDV P protein

CDV P cDNA was cloned from the CDV Yanaka strain infected cells [Wakasa, Genbank accession no. AB028914]. CDV P gene produced three kinds of viral proteins, P, V, and C [Lamb and Kolakofsky, 2001]. The V protein shares its N-terminal half (1-231 aa) with the P protein and has a different C-terminus (232-246 aa). Therefore, a DNA fragment coding C-terminal half of P protein (232 aa to 507aa) was used as the antigen. The DNA fragment was inserted into E. coli expression vector pQE30 (QIAGEN) which can produce recombinant protein as a fusion protein with histidine-tag at N-terminal. The plasmid was transformed in the M15 bacteria strain for high-yield of recombinant proteins. One-litter culture was incubated to express CDV-P at mid-log phase of growth (0.2 OD_{600}) by addition of 1mM IPTG. After 3 hr incubation, the E. coli culture were centrifuged, pelleted and washed by PBS (-). It was lysed by lysis buffer (0.5% Triton X-100, 0.5% DOC, 5mM NaCl, 25mM Tris-HCl pH8.0) and centrifuged at 10,000xg for 30 min to remove insoluble materials. Recombinant CDV-P was found mainly in soluble

fractions. The P protein containing the N-terminal histidine tag was purified according to chapter 3. This purified recombinant P protein was provided as an antigen for generation of MAbs.

Generation of monoclonal antibodies

The recombinant CDV P protein was mixed with RIBI adjuvant (Corixa) and immunized into BALB/c mice three times. Anti-CDV P antibody titer was checked by ELISA using the recombinant P protein as antigen. When high titer of P antibody in these sera was detected, mice were sacrificed, and then spleen cells were obtained and fused with myeloma PAI cells using PEG1500 (Roche) as described previously [Kohler and Milstein, 1975]. Hybridoma cells were selected by HAT (Gibco, BRL) and culture supernatants were examined by immunofluorescence (IFA) using B95a cells infected with the CDV Yanaka strain. Hybridomas with positive reaction were selected, propagated and inoculated into peritoneal cavity of BALB/c mice after pristan treatment (Sigma-Aldrich). After approximately 10 days, ascites including high titer of MAbs were collected.

Immunofluorescence assay (IFA)

B95a cells were grown in 10cm plates and infected with various strains of CDV, RPV and MV. After 3 days, the cells were removed by scraper, washed once with PBS (-) and cultured in 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 the MAbs (diluted at 1:200) in PBS (-) washed, and then goat anti-mouse antibody- FITC conjugate in PBS (diluted at 1:500) (CAPPEL). After washing by PBS three times, imaging analysis was

performed with confocal microscopy, Fluoview FV500 system (Olympus).

Immunoprecipitation

B95a cells infected with or without the Yanaka strain were radiolabelled with 3 MBq/ml [^{35}S] methionine and cystine (DuPont NEN Research Products) for 2 hrs. The radiolabelled cells were lysed in RIPA buffer (1% Triton X-100, 1mM iodoacetamide, 0.2 U/ml of the trypsin inhibitor aprotinin, 1mM phenylmethylsulfonyl fluoride, 1% sodium deoxycholate, 0.14M NaCl, and 10mM Tris-HCl, pH 8.0). The lysate was reacted with the MAbs for 3 hrs at 4°C, followed by incubation with protein A beads (Amersham Biosciences). The beads were washed with PBS (-) three times and then loading dye was directly added. The immunoprecipitates were resolved by 10% SDS-PAGE and analyzed by BAS2000 (Fuji Film).

Competitive binding inhibition assay

MAbs were purified by protein A sepharose and then biotinylated MAbs were prepared according to the manufacture procedure (Biotin labeling kit, Roche). CDV-Yanaka infected B95a cells were lysed by RIPA buffer and the lysate was spread in each well of 96-well plate. After 1 hr at 37℃, each well was blocked by PBS with 4% block ace. Each well was reacted with appropriate dilutions of non-labeled MAbs for 1 hr at 37℃ and then washed five times with PBS. Each well was reacted with appropriate dilutions of each biotinylated MAbs and washed as well as described above. The detection was performed by Vectastain Elite ABC kit (Vector Laboratories). After the reaction was visualized with o-phenylenediamine and then stopped with H_2SO_4, the resulting optical density at 492 nm was measured with an ELISA reader (Bio-Rad).

RESULTS

Production of Recombinant P Protein derived from the CDV Yanaka strain.

CDV P gene produced three kinds of viral proteins, P, V, and C [Lamb and Kolakofsky, 2001], and the P protein (507 aa) is expressed from the largest ORF in the P mRNA (Fig. 1A). The V protein (299 aa) shares its N-terminal half with the P protein and has a different C-terminus. To produce P protein specific antibodies, we prepared a DNA fragment coding C-terminal region of P protein of the CDV Yanaka strain (232 aa to 507aa). The DNA fragment was inserted into E.coli expression vector which recombinant protein was expressed as a fusion protein with histidine tag at N-terminal. E.coli transformed with the plasmid produced recombinant P protein in soluble form and by single-step affinity purification procedure using nickel chelating column. The purified protein was shown with an expected molecular weight of approximately 21 kDa and obtained at high yield (Fig. 1B). The purified CDV-P protein was applied for the generation of antibodies.

Establishment of CDV-P MAbs against CDV P protein

Author immunized the recombinant CDV P protein in mice and fused their splenocytes to myeloma cells to establish hybridomas. After first screening by ELISA using recombinant CDV-P protein, 205 positive-colonies were obtained. Following screening by ELISA using lysate of B95a cells infected with the CDV Yanaka strain, author selected seven clones, 13Ea, 52G, 36Ba, 33Ba, 36E 42Ba, and 99Bb, which hybridoma cells were propagated well and their supernatant showed high reactivities to P protein of the CDV Yanaka strain. In analyses of IFA and immunoprecipitation, positive reactivity of all of the MAbs reacted with B95a cells infected with the CDV Yanaka strain

was observed and specific 72kDa-bands were visualized respectively (Fig. 2). Although immunoprecipitation by some anti-N MAbs was respected to be co-precipitated due to N-P interaction [Orvell, 1985; Sugiyama, 1989], none of the related proteins was precipitated by all the anti-P MAbs (Fig. 2). No MAbs reacted with V and C proteins of the CDV Yanaka strain transiently expressed in COS1 cells (data not shown). These results indicate that the MAbs specifically react with P protein of the CDV Yanaka strain.

Characterization of MAbs

Antigenic sites recognized by the 7 MAbs were determined by a competitive binding inhibition assay using the lysate of B95a cells infected with the CDV Yanaka strain (Table 1). The analysis revealed that the MAbs recognized in two different epitopics of P protein. MAb 13Ea, 52G, 36Ba, 42Ba, and 99Bb reacted one epitope (site I), and 33Ba and 36E another (site II).

Reactivity of the MAbs against Morbilliviruses

The 7 MAbs were examined for their antigenic specific reactivity against morbillivirus strains; the CDV Onderstepoort, CDV-Snyder Hill, CDV-Haku 93 and Haku 00, RPV-RBOK, RPV-L, MV-Edmonston and MV-HL strains (Table 2). New field isolates of CDV, the CDV Haku93 and Haku00 strains, showed reactivity to the MAbs recognizing antigenic site I, 13Ea, 52G, 36Ba and 99Bb, but they did not react with the MAbs for site II, 33Ba and 36E. Unexpectedly, all of the MAbs did not react with both of old type CDV strains; the Onderstepoort and Snyder Hill strains.

All MAbs did not show positive reactivity with any of MV infected cells. Interestingly, MAbs for site I reacted with RPV infected cells except MAb 99Bb. The P

protein of RPVs probably conserved the antigenic site I, but some genetic alteration might cause minor differences of antigenicity which is responsible for the MAb 99Bb recognition.

DISCUSSION

In this study, author produced the specific region (321-507aa coding) recombinant P protein of the CDV Yanaka strain by E.coli and established seven hybridomas producing MAbs against the protein. The seven MAbs recognized two antigenic epitopes (site I and II). Orvell and colleagues reported that the established MAbs against whole P protein of the CDV Convac strain had 6 epitopic groups in 22 MAbs [Orvell, 1985]. P protein in RNP is proposed to form tetramers with helical coiled coils in the central region (approximately 340-410aa) and with a compact bundle of three alpha-helices for N-binding at the C-terminal region (457-507aa of MV) [Kingston, 2004]. The tetrameric P protein structurally penetrates to L protein at the central region of P protein and thus the region is masked due to interaction of L protein. Thus by the screening method of IFA using CDV infected B95a cells, it is speculated that MAbs recognized N-binding region are more selective than L-binding region. All the anti-P MAbs did not induce to co-precipitate CDV-N-proteins. This result suggests that the antigenic region of the MAbs might locate at the region of N-P interaction.

Sequence alignment of the P protein of the Yanaka strain with that of the Onderstepoort strain showed 94% identity [Genbank accession, Yanaka: AB028914; Onderstepoort: AF378705]. Highly conserved regions were preferentially located in the C-terminal half corresponding to the recombinant P protein and were 96% identical to each other. Although the P gene sequences of Haku93 and Haku00 were not determined, those of H proteins showed more than 98% similarity to the Yanaka strain, indicating high similarity also for P protein. On the other hand, the amino acid identities between the CDV Yanaka strain and other morbilliviruses are only 44.3% (MV Edmonston) and 46.0% (RPV-RBOK). Curiously, the MAbs, 13Ea, 52G, 36Ba and 42Ba,

reacted with the RPV-RBOK and RPV-L strains but neither the CDV Onderstepoort nor Snyder Hill strains, probably recognizing a conformation-dependent epitope. This result may be explained by previous study that one anti-CDV-P MAb is found with reactivities of the CDV Onderstepoort and Convac strains but not the CDV Rockborn strain. And another study also showed that one anti-MV-M MAb cross-reacted with the CDV Convac and RPV RBOK strains but not the CDV Onderstepoort strain, and another MAb reacted with only the RPV RBOK strain but not the CDV Convac and Onderstepoort strains [Sheshberadaran, 1986]. All proteins consist of both functional highly conservative regions and non-functional variant regions. These facts raise a possibility that one of the conservative regions in P protein was evolutionally changed independently, and then a common epitopic structure in P proteins abiogeneticly appeared in both the CDV Yanaka and PRV strains.

In this study, MAbs of P protein of CDV Yanaka atrain established in this study should be useful for functional study of P protein.

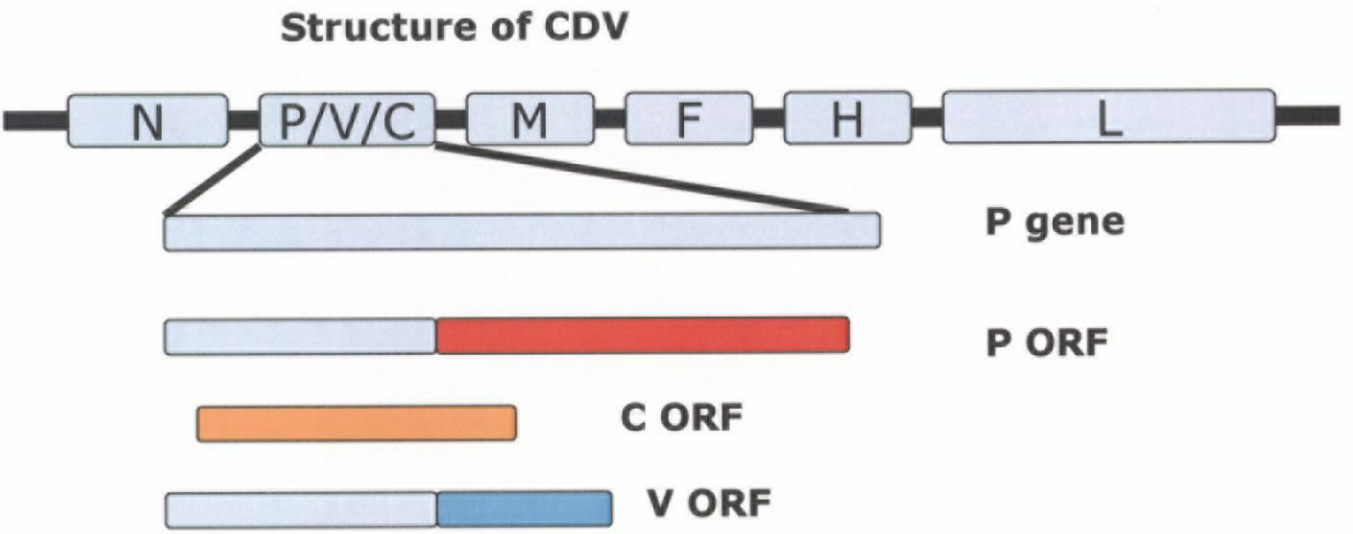
FIGURE LEGENDS

Fig. 1 Expression of recombinant P protein. (A) Scheme of CDV-P gene. CDV P gene produced three kinds of viral proteins, P, V, and C. (B) Sliver staining (left) and western blot analyses (right) are shown. The P protein containing the N-terminal histidine-tag purified with NTA resin column was observed as a single band (arrow). 1. E.coli, 2. prepurification, 3. purified CDV-P protein.

Fig. 2 Immunoprecipitation by each MAb against CDV-P protein.
CDV-Yanaka infected B95a cells were labeled with ^{35}S-Met and Cys, and immunoprecipitated by MAb-Protein A Sepharose. Expected size of protein bands were observed at 72kDa (indicated by arrow).

Fig. 1

A)


Structure of CDV
N
P/V/C
M
F
H
L
P gene
P ORF
C ORF
V ORF

B)

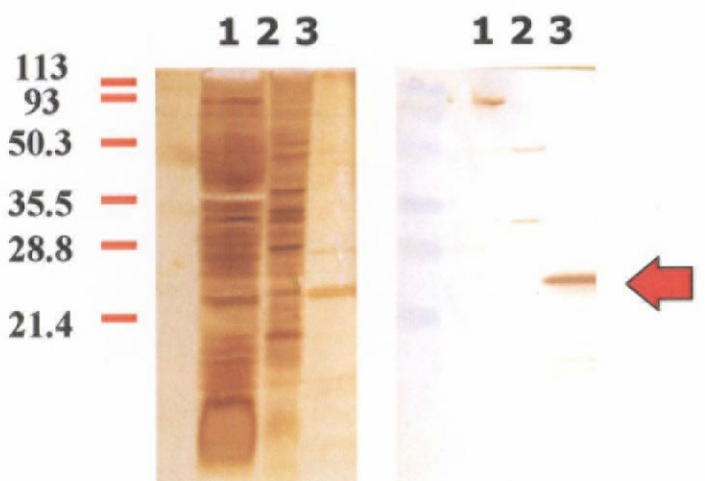

1 2 3
1 2 3
113
93
50.3
35.5
28.8
21.4

Fig. 2

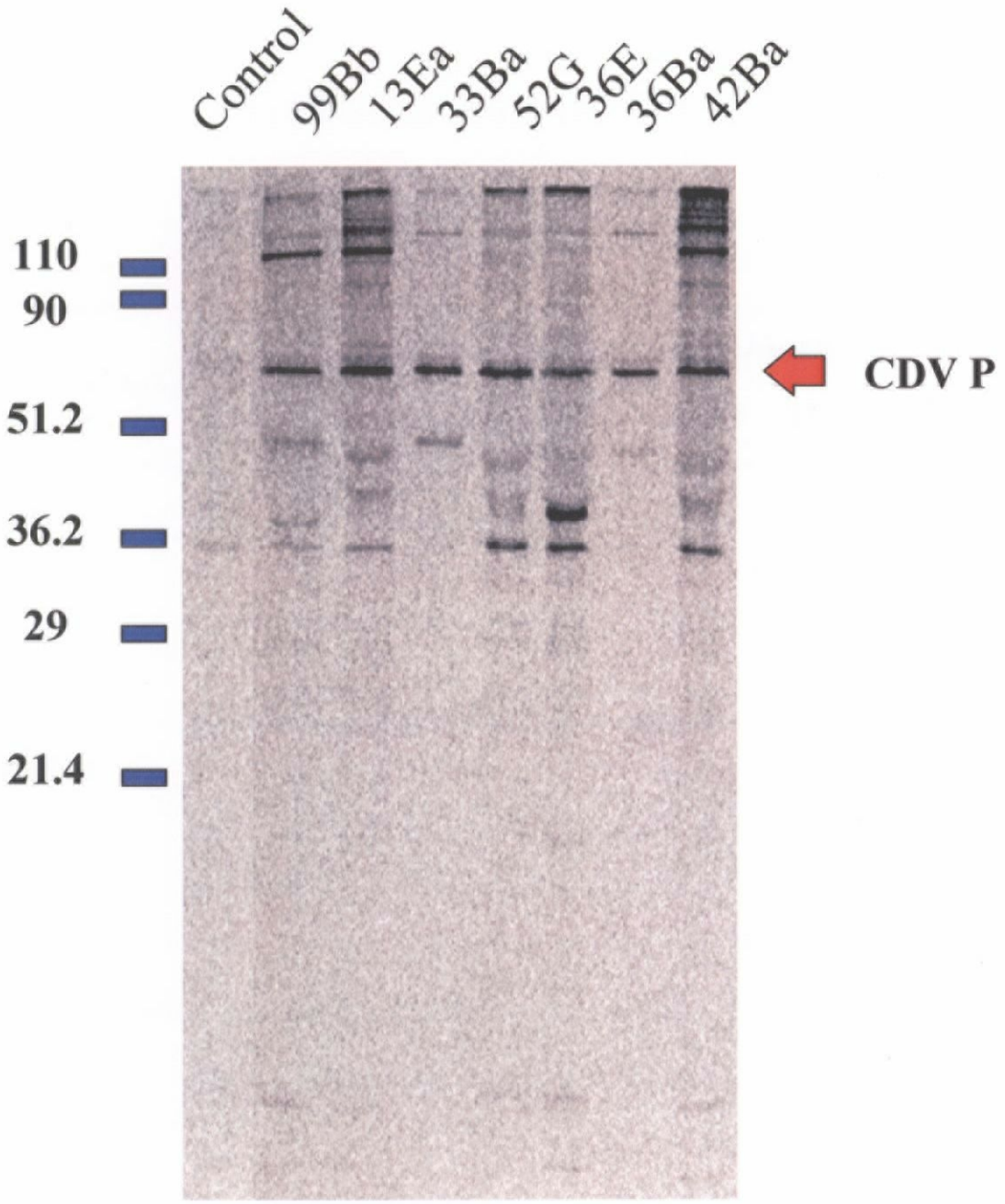

Control
99Bb
13Ea
33Ba
52G
36E
36Ba
42Ba
110
90
51.2
36.2
29
21.4
CDV P

Table 1 Competitive binding inhibition assay

Purified MAbs	Antigenic site	Biotinylated MAb						
		99Bb	13Ea	52G	36Ba	42Ba	33Ba	36E
99Bb	I	+	+	+	+	+	-	-
13Ea	I	+	+	+	+	+	-	-
52G	I	+	+	+	+	+	-	-
36Ba	I	+	+	+	+	+	-	-
42Ba	I	+	+	+	+	+	-	-
33Ba	II	-	-	-	-	-	+	+
36E	II	-	-	-	-	-	+	+

* +: 80%< competition, -: 30%> competition

Table 2 Immuno reactivity of anti-P MAbs with various strains of morbilliviruses

		MAb						
		99Bb	13Ea	52G	36Ba	42Ba	33Ba	36E
CDV								
	Yanaka	+	+	+	+	+	+	+
	Onderstepoort	-	-	-	-	-	-	-
	Snyder Hill	-	-	-	-	-	-	-
	Haku93	+	+	+	+	+	-	-
	Haku00	+	+	+	+	+	-	-
RPV								
	RBOK	-	+	+	+	+	-	-
	L	-	+	+	+	+	-	-
MV								
	Edmonston	-	-	-	-	-	-	-
	HL	-	-	-	-	-	-	-

CHAPTER 5

Characterization of CDV Genome in Viral Particle by Electron Microscopy

SUMMARY

Compared to the studies of CDV or MV genomes released in cytoplasm of infected cells, morphologic studies of them in virus particle remain unknown. In this study, author investigated the morphologic features of CDV genome packaged in viral particle by electron microscopy. The released CDV particles were prepared from culture medium by sucrose gradient ultra-centrifugation. In electron microscopic analysis, viral genome packaged in viral particle was extensively longer than predicted in length (1000 nm). Additionally, the viral genomes existed as large filamentous (LF) form and small filamentous (SF) form. By immunoelectron microscopic analysis using the monoclonal antibodies against CDV P proteins established in Chapter 4, P protein was only localized on the surface of genome as LF form of genome but not as SF form. These results suggest that multiple genomes are packaged in a single viral particle and P protein-attached filamentous structure is a natural form in infectious viral particles.

INTRODUCTION

Paramyxoviruses have a single-stranded non-segmented negative-sense RNA genome of between 15 and 19kb in length [Lamb and Kolakofsky, 2001]. The RNA genome is packaged with the viral nucleoprotein (N) within a helical nucleocapsid. *Mononegavirales* use this N-RNA complex (here after referred to as the nucleocapsid) as a template for both transcription and replication. These reactions are carried out by the RNA-dependent RNA polymerase (L) in conjunction with the phosphoprotein (P). The morphology of nucleocapsid is considered as one of the defining features of the *Paramyxoviridae* and is described as having a 'herringbone' appearance when imaged under a transmission electron microscope. The nucleocapsid, rather than naked genomic RNA, is the template for replication and transcription [Emerson, 1987]. The nucleocapsid is packaged in viral particle with viral glycoproteins and then released from the cells. Compared to the nucleocapsid in the intracellular environment, its morphologic structure in viral particle has not been well-investigated. Although the nucleocapsid penetrates cytoplasm after the infection, it is not clear how adequate morphologic structure of nucleocapsid is packaged in a viral particle in order to start transcription and replication immediately and where P and L proteins are localized in viral genome for transcription and replication.

In this study, to investigate morphologic structure of packaged CDV genome in viral particle, author purified CDV particles from culture medium by sucrose gradient ultra-centrifugation, and performed electron microscopic analysis. Furthermore, author determined localization and distribution of CDV P protein in the genome by immunoelectron microscopy using the MAbs against CDV P protein established in Chapter 4.

MATERIALS AND METHODS

Cells and viruses

B95a cells, which were derived from a marmoset B-lymphoblastoid cell line were maintained in RPMI1640 containing 5% FCS. The CDV Yanaka strain was isolated in Japan from a dog clinically diagnosed with distemper [Gemma 1996b].

Electron microscopic analysis

B95a cells were infected with the CDV Yanaka strain at a MOI of 0.1, and 2 days later the cells were fixed in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH7.3) for 1 hr. The cells were post-fixed in 1% osmium in the phosphate-buffer for 1 hr, dehydrated and embedded in Epon (TAAB). Fifty μm of ultra thin sections were prepared by ultra microtome (Leica) and stained with 2% Urinaly acetate in 70% Ethyl alcohol and lead citrate. The sections were examined with 1200EX electron microscope (Jeol). Culture medium collected from B95a cells infected with the CDV Yanaka strain was concentrated by filtration system (Centricon filter, pore size of 100K, Millipore). The concentrate was applied to sucrose bed at the dense between 20% and 60% and separated by centrifugation with 25,000 x g for 2 hr. Virus particle-including band was purified by desalting column in order to remove the sucrose. The samples were dropped on the plastic-coated copper grids and incubated for 15 min at room temperature. The viral particles just after budding were also examined with electron microscopy (Jeol).

Immunoelectron microscopic analysis

The purified viral particles were dropped on the plastic-coated copper grids and

incubated as described above. After blocking with 3% gelatin in PBS (-) for 1 hr, the sample was incubated with anit-CDV P MAb (36Ba), CDV-N MAb (6H) or CDV-M polyclonal antibody [Uema, 2005] for 1 hr at room temperature. After three times washing with PBS (-), the sample was labeled by gold particle-protein A (Amarsham Bioscience) for 1 hr. After several wash by 0.05M phosphate buffer, grids were post-stained by 2% phosphotangstic acid. Immunoelectron microscopic analysis was demonstrated by 1200EX (Jeol).

RESULTS

Electron microscopic analysis of CDV Yanaka infected B95a cells

In previous electron microscope studies, viral formation or budding of CDV or MV was characterized in Vero cells or Hela cells [CDV: Narang, 1982; MV: Nakai, 1969; Oyanagi, 1971]. Initially, author investigated how viral particle of CDV released from CDV-Yanaka infected B95a cells (Fig. 1AB). Viral particles near the surface of a cell are clearly visible. One spherical viral particle was observed near the cell (Fig. 1A a) and other particles have attached to the cells and the interior of the particle was continuous with the cytoplasm (Fig. 1A b, c). The shedding of viral particles was always accompanied with extensive spiking. On the other hand, nucleocapsid regularly aggregated in cytoplasm (Fig. 1B, NC). These results agreed with the previous studies [Dubois-Dalcq, 1987].

Electron microscopic analysis of structure of CDV genome in virus particle

To investigate the genomic structure packaged in the virus particle of the CDV Yanaka strain, released viral particles from B95a cells infected with the virus were concentrated and separated by sucrose gradient centrifugation. The virus particle and its genome were observed under electron microscope (Fig. 2). As expected, the viral particle displayed a relatively homogenous population of spherical particles with a diameter of around 200nm (Fig. 2AB), as described previously [Lamb and Kolakofsky, 2001]. Typical nucleocapsid strands of CDV genome were also clearly seen. Unpackaged genome was observed probably due to disruption of the virus particle (Fig. 2C). Partially, a smaller diameter of nucleocapsid string was observed (Fig. 2C arrow). Based on the scale bar (100 nm), the length of the genomes was estimated. The genome length in a

viral particle was estimated to be more than 2000-3000 nm in length. Since the size of a single genome of paramyxovirus described as approximately 1000 nm in length previously [Lamb and Kolakofsky, 2001], the possibility that multiple genomes are packaged in a single virus particle was raised.

Immunoelectron microscopic analysis of CDV genome by anti-N, P and M antibodies

Using each antibody against P, N or M protein of the CDV Yanaka strain, its localization and distribution on the CDV genome was observed by immunoelectron microscopy. The genomes reacted with the CDV-P MAb, CDV-N MAb or CDV-M polyclonal antibody. The antibody reaction was visible as black dots. The genome in unfixed viral particle was not able to react with the antibodies, because the viral membrane probably interfered with these reactions. Under the electro microscopy, the viral genomes with filamentous structure apparently consisted of two forms with diameters of approximately 80 nm and 20 nm. Author designated large filamentous (LF) and small filamentous (SF) genome (Fig. 3, Fig. 4B). The diameter of typical paramyxyovirus genome was described as 18 nm [Lamb and Kolakofsky, 2001], corresponding to the SF genome. Anti CDV P MAb reacted with only LF genome but not SF genomes, and the reaction to the LF genomes occurred over the whole strings (Fig. 3A-C). Anti-N MAb reacted with both LF and SF genomes, implying that the strings are nucleocapsids (Fig. 4A, B). The analysis using anti-M antibody was also carried out. M proteins existed interspersely (Fig. 5 A, B) and thus did not attach to the genome.

DISCUSSION

In this study, author shows that viral genome packaged in a viral particle was extensively longer than previous predicted size. Additionally, the viral genomes existed as two forms, LF form and SF form. By immunoelectron microscopic analysis, P protein was only localized on the surface of genome of LF form but not SF form.

A paramyxovirus produces pleomorphic particles with diameters ranging from 120 to over 300 nm [Waterson, 1965; Nakai, 1969; Lund, 1984]. The cargo space of the particles is speculated to vary from 3×10^5 to $>10^7$ nm^3. Considering that the diameter and length of the virus genome are 20 and 1000 nm, respectively, particles should be able to accommodate from one to >30 genomes. Actually, particle sedimentation and ultraviolet inactivation studies indicate that the pleomorphic particles of parainfluenza viruses often incorporate more than one genome [Hosaka, 1966; Dahlberg, 1969] and previous studies of recombinant MV demonstrated that multiple heterogenous genomes are packaged stably in a virus particle and the particles are produced in standard infection at no expense to infectivity [Rager, 2002]. This electron microscopic analysis shows that a lot of both packaged and unpackaged viral genomes were extremely longer than the expected length of virus genome. This result suggests that packaging of multiple genomes generally occurred in many virus particles and thus the multiple genomes can effectively start to induce the transcription and replication in infected cells.

Our observation showed that the two forms with various diameters of genomes existed. The SF form was closely identical to the general morphology shown in previous studies in MV [Nakai, 1969]. On the other hand, as observed by immunoelectron microscopic analysis with anti-P and anti-N antibodies, LF form seemed to be based on SF form as a helical core and covered with P proteins together with N proteins. This

result seems that the filamentous structure covered with P proteins is a natural form in viral particle, possibly for the rapid start of transcription and replication. In case of extensive expression of N proteins at the condition without genomic RNA, filamentous string structure is known to be formed which is similar to the SF form [Schoehn, 2004]. Taken together, newly synthesized viral genome is rapidly coiled and simultaneously covered by N proteins, and then the virus genome covered with N and P proteins may functionally start transcription and replication.

FIGURE LEGENDS

Fig. 1 Electron microscope of B95a cells infected with the CDV Yanaka strain. The cells were stained with 2% Urinayl acetate in 70% Ethyl alcohol and examined by electron microscopy. (A) Cells infected with CDV-Yanaka. Arrow heads indicate viral particles released from the surface of the infected cells. (B) Intracellular feature of infected cells. Nucleocapsid (NC) aggregates in cytoplasm.

Fig. 2 Electron microscope of released viral particles of the CDV Yanaka strain.
(A, B) CDV-virions. Filamentous genome is packaged in the virion. (C) Naked genome of CDV. Arrow head indicates small filamentous genome.

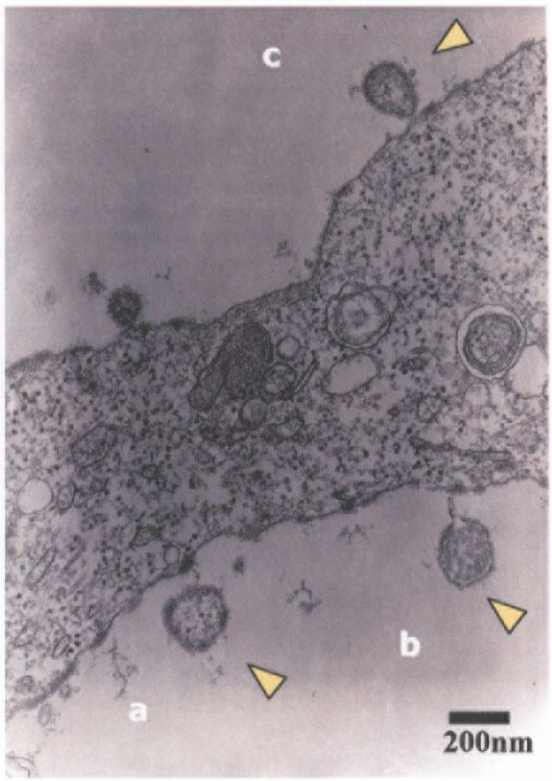
Fig. 3 Immunoelectron microscope of CDV genome by anti-CDV P antibody (A-C). Arrow head indicates small filamentous genome (B, C).

Fig. 4 Immunoelectron microscope of CDV genome by anti- CDV N antibody (A, B). Arrow head indicates small filamentous genome.

Fig. 5 Immunoelectron microscope of CDV genome by anti- CDV M antibody (A, B).

Fig. 1

(A)



(B)

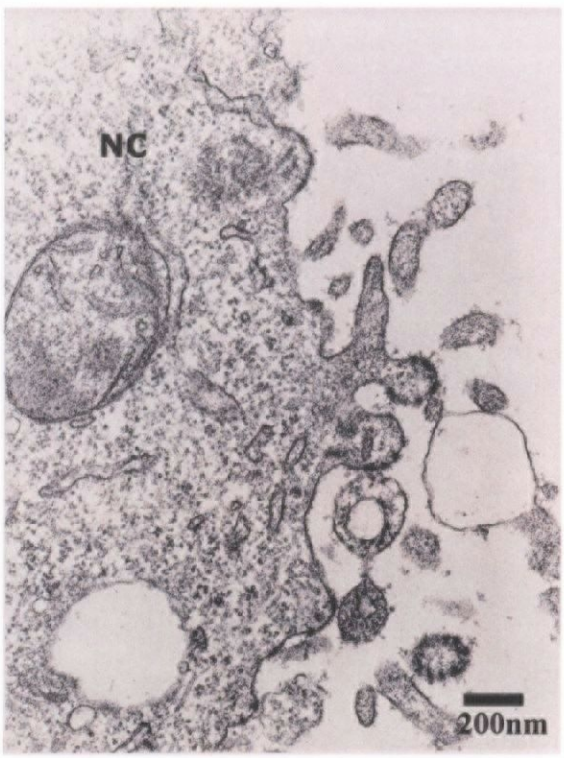
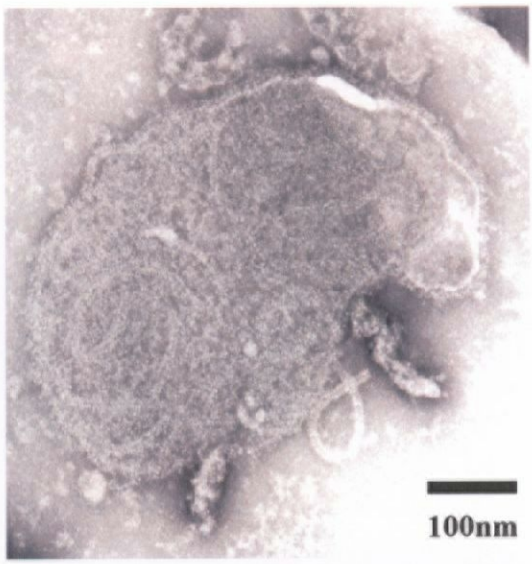
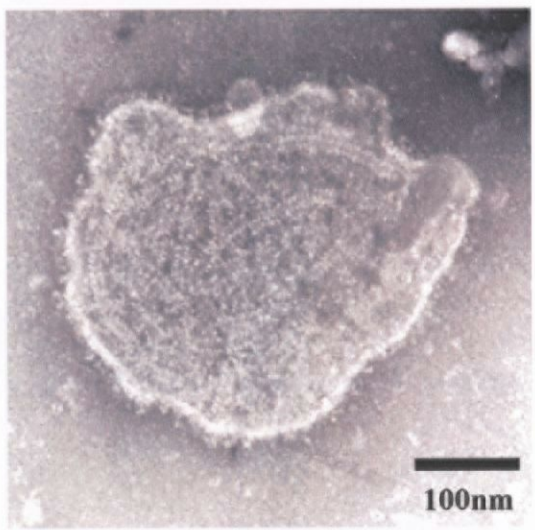


Fig. 2

(A)



(B)



(C)

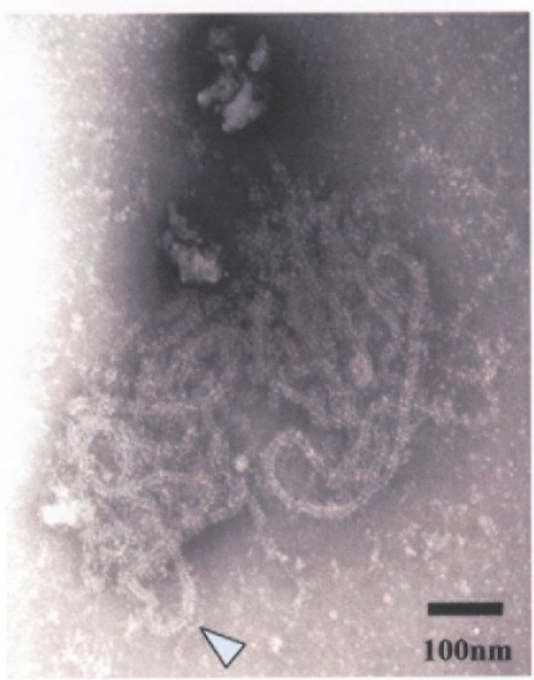
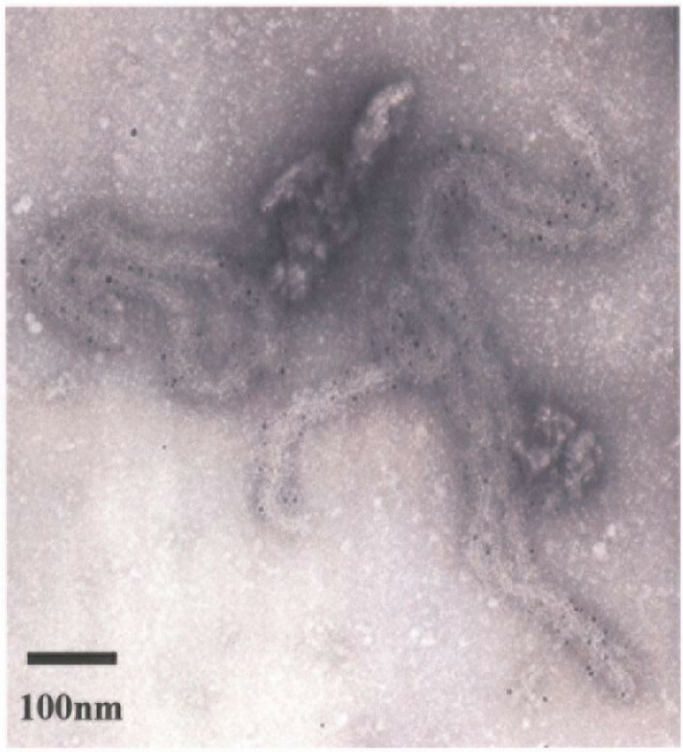
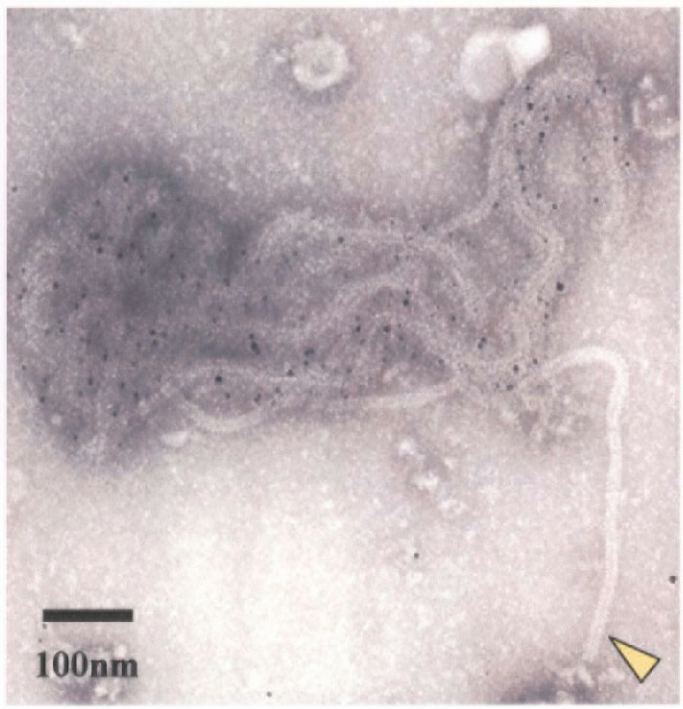


Fig. 3A, B

(A)

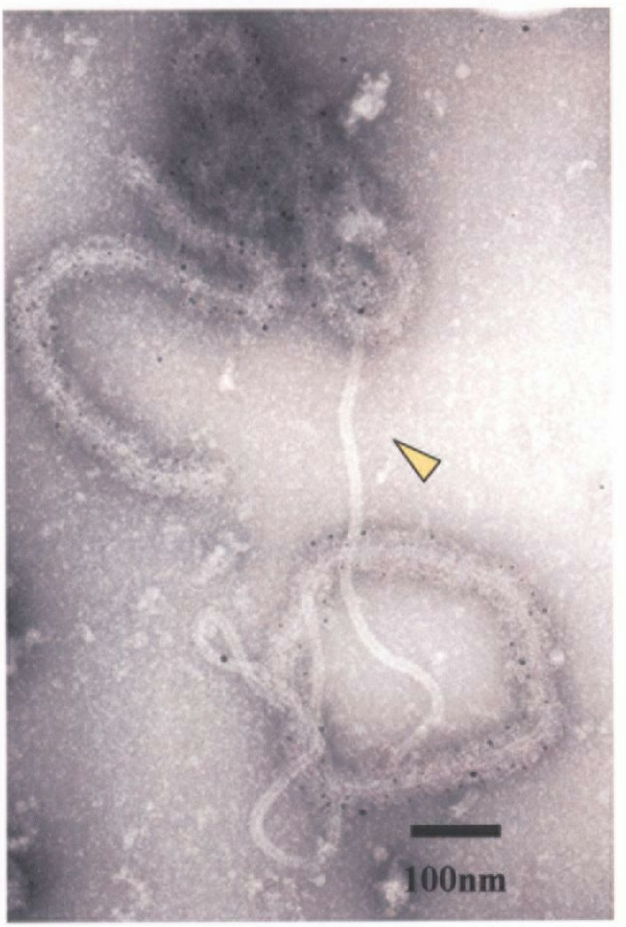


(B)



Anti-P MAb

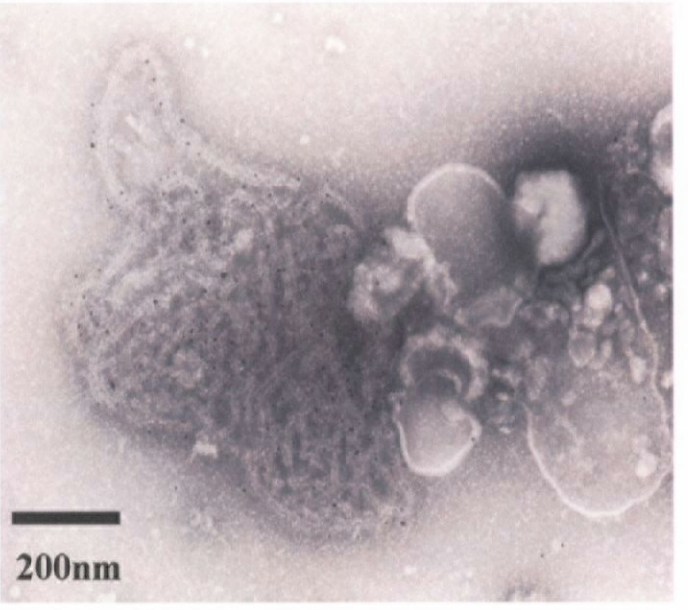
Fig. 3C



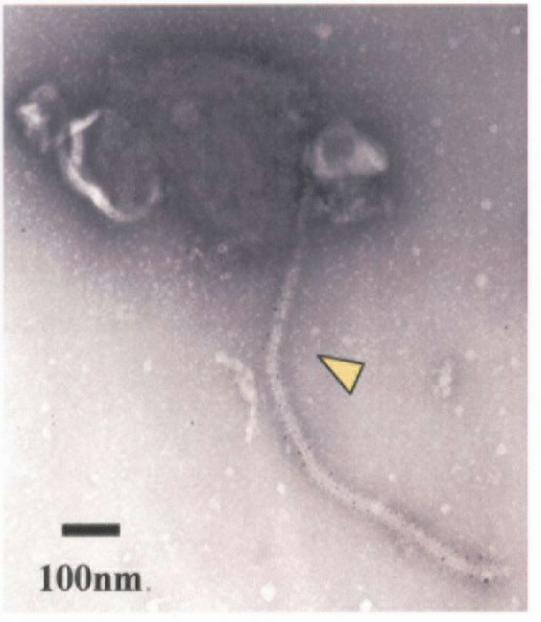
Anti-P MAb

Fig. 4

(A)



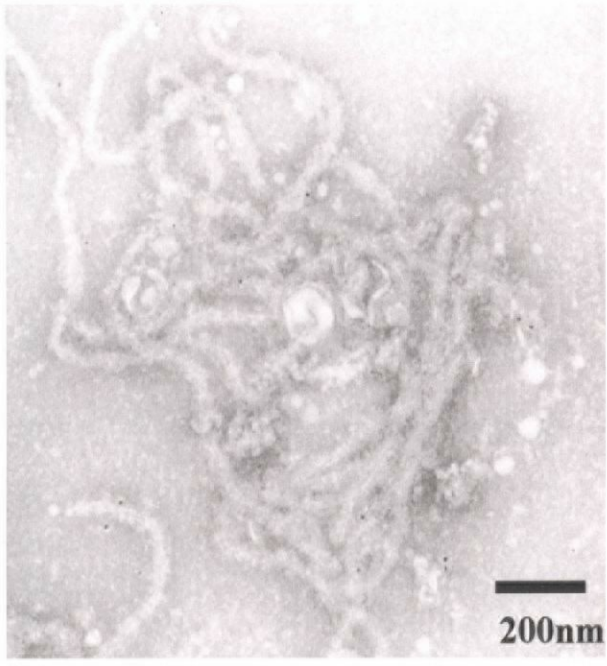
(B)



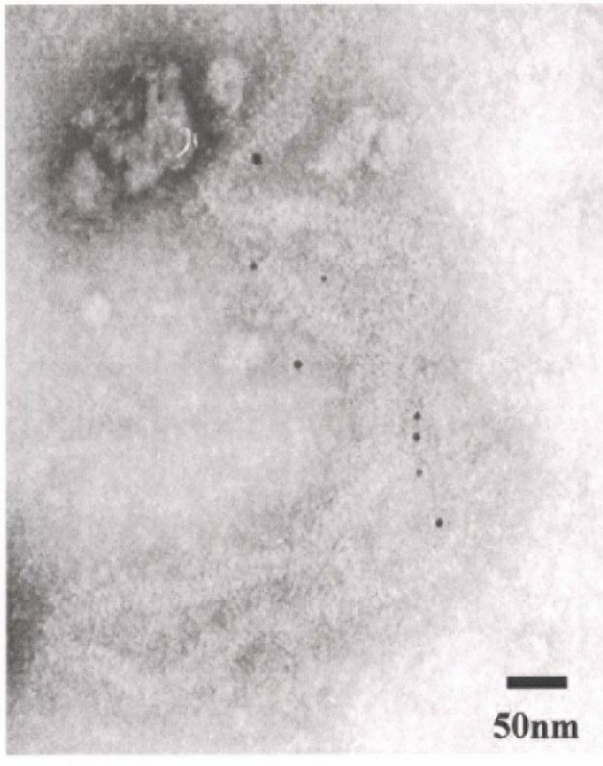
Anti-N MAb

Fig. 5

(A)



(B)



Anti-M MAb

CONCLUSION

Mononegavirus with reverse genetics system is a useful tool not only to analyze factors involved in pathogeny, tissue tropism and viral transcription and replication, but also to generate recombinant vaccines, gene expression vectors and gene delivery vectors. Using the CDV reverse genetics system, the author aimed in this thesis to understand the mechanism of CDV propagation and spread in dog hippocampus and to develop the therapeutic CDV vectors and leishmania polyvalent vaccine based on CDV. In addition, author also aimed to obtain the fundamental information of genomic structure of CDV to study the biological characteristics of infection and replication.

In Chapter 1, the author analyzed the mechanism of CDV propagation and spread in dog hippocampal slices by using recombinant CDV expressing enhanced green fluorescence protein (EGFP-CDV). The CDV infection and propagation mainly occurred in cornus ammonis regions. Neurons are more effectively infected with the EGFP-CDV than astrocytes and oligodendrocytes. The EGFP-CDV was observed to transport from a neuron to the other through synapses, and a part of synapses of infected neuron mediated the transmission. The viral envelop proteins F and H were distributed in the whole neurons but M protein is mainly located at soma and partially at dendrites and synapses. Electron microscopic analysis demonstrated that ribonucleocapsid protein (RNP) is sorted to synaptic terminals and viral particles were visualized at extrasynaptic regions. These data suggest that CDV may be transmitted to synapses of neurons via extrasynaptic regions. This study shows that CDV is available for long-term transduction of neurons as a delivery vector.

In chapter 2, the author constructed a recombinant CDV expressing dog superoxide dismutase 1 (SOD1) and characterized the effect of recombinant CDV. Since

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. 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 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 cells and the recombinant CDV is considered to be useful as a delivery viral vector.

In Chapter 3, the author displayed recombinant CDVs for use as effective polyvalent vaccine candidates against CDV and Leishmania infections. Two recombinant CDVs expressing Leishmania antigens, a homologue to eukaryotic thiol-specific antioxidant protein (TSA) and a homologue of a yeast stress-inducible protein 1 (LmSTI1) were successfully rescued (TSA-CDV and LmSTI1-CDV). Dogs immunized with the TSA-CDV, LmSTI1-CDV or the mixture of TSA-CDV, LmSTI1-CDV and LACK-CDV did not show observable clinical signs of infection. The dogs were challenged with *Leishmania major* intradermally in the ears. 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 in all stages of *leishmania* infection. The results indicate that the recombinant CDV with *Leishmania* antigens could induce protective immunity in the inoculated dogs against both CDV and *Leishmania infections*. This study shows

that the recombinant CDVs are powerful tools for the prevention of leishmaniasis epidemics.

In Chapter 4, the author produced monoclonal antibodies (MAbs) against CDV P protein in order to analyze the functions of P protein. P gene of the CDV Yanaka strain, not V nor C region, was cloned into pQE E.coli expression vector, and P protein was produced in mass culture and purified. By immunizing the expressed protein into BALB/c mice, author obtained 7 monoclonal antibodies (13Ea, 52G, 36Ba, 33Ba, 36E 42Ba, and 99Bb). Competitive binding assay revealed that they recognized two antigenic sites of P protein. The first site was interestingly conserved in other recent field isolates and in RPVs, but not in old type CDVs nor in MVs. The second site was appeared specifically only for the Yanaka strain. MAbs of P protein of the CDV Yanaka strain established in this study should become a useful tool for functional study of P protein.

In Chapter 5, the author investigated the morphologic features of CDV genome packaged in viral particle by electron microscopy. The released CDV particles were prepared from culture medium by sucrose gradient ultra-centrifugation. In electron microscopic analysis, viral genome packaged in viral particle was extensively longer than predicted in length (1000 nm). Additionally, the viral genomes existed as large filamentous (LF) form and small filamentous (SF) form. By immunoelectron microscopic analysis using the MAbs against CDV P proteins established in Chapter 4, P protein was only localized on the surface of genome as LF form of genome but not as SF form. These results suggest that multiple genomes are packaged in a single viral particle and P protein-attached filamentous structure is a natural form in infectious viral particles.

These finding described in this thesis supplied invaluable knowledges of CDV infection tropism in dog' brain and morphologic features of the genome, and showed

high availability of the recombinant CDV as a novel viral vector for gene therapies and polyvalent vaccines.

ACKNOWLEDGMENTS

I would like to express my gratitude to everyone who has contributed to this thesis, in particular to:

Professor Chieko Kai (Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, Tokyo) for invaluable support, critical encouragement and excellent scientific advice.

Lecturer Kyoko Tsukiyama-Kohara (Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, Tokyo), for excellent scientific and technical advice.

Research Associate Ryuichi Miura (Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, Tokyo), for excellent scientific and technical advice.

Research Associate Yoneda Misako (Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, Tokyo), for excellent scientific and technical advice.

Research Associate Sagara Hiroshi (Laboratory of molecular structure analysis, Division of Fine Morphology, Fine Morphology Laboratory, The University of Tokyo, Tokyo), for encouragement, and excellent scientific and technical advice.

Lecturer Endo Yasuyuki (Laboratory of Internal Medicine, Department of Veterinary Medicine, Faculty of Agriculture, Kagoshima University, Kagoshima, Kagoshima), for encouragement.

Dr. Kentaro Fujita (Department of Molecular Genetics and Microbiology, State University of New York at Stony Brook, New York, USA), for excellent scientific and technical advice.

The colleagues of all members, of Laboratory Animal Research Center,

Institute of Medical Science (The University of Tokyo, Tokyo), in particular to: Dr. F. Kobune, Dr. Kanai, F. Ikeda, Y. Goto, A. Takenaka, Dr. A. Nuntaprasert, Dr. K. Togashi, Dr. H. Sato, Dr. Y. Katakai, Dr. T. Kuraishi, Dr. Y. Fukushima, Dr. A. Watanabe, Dr. Y. Inoue, Dr. K. Hirabayashi, Dr. Y. Muto, R. Sato, T. Katsuo, M. Tomoeda, Y. Sakuma, R. Takehara, Y. Okamoto, M. Seto, M. Katayama, K. Iwasaki, M. Kurita, M. Doki, M. Uema, M. Shiotani, T. Seki, K. Hirama, T. Nishi, M. Kanai, M. Izumi, M. Hoshi, C. Imai, T. Takano, M. Funabashi, M. Masuda, T. Kubota, K. Saito, M. Awakuni, K. Fukumoto, M. Huang, R. Fukaya, H. Uema and T. Kidokoro for many technical advices and refreshing time.

All of my friends in Institute of Medical Science (The University of Tokyo, Tokyo), in particular to: Dr. T. Ijyuin and Dr. H. Sasaki in Division of Biochemistry, Department of Cancer Biology, Dr. K. Masubuchi, Yamamoto, M. Fujimoto, Y. Yamauchi, Dr. M. Watanabe and Dr. M. Murakami in division of genetics, T. Miyasaka and M. Goto in division of oncology, Dr. Matsumura in Division of Cellularand Molecular Biology, Department of Cancer Biology; Prof. Y. Nakamura, Dr. Oguro, Dr. Fujiwara, H. Sato, C. Colin, Hara, Ms. Takada, H. Kodama and Y. Watanabe in Division of Molecular Biology, Dr. T. Kurita in Division of Molecular and Developmental Biology, T. Kimori and M. Shiraishi in Laboratory of molecular structure analysis, Division of Fine Morphology, department of Basic Medical Sciences; H. Iwai, Nagai, M. Suzuki, A. Ishijima, A. Yoshii, in Division of Bacterial Infection, T. Noda, M. Muramoto, T. Fujii and Dr. Takada Division of Virology department of Microbiology and Immunology; Urano and Minoshima in Division of Hematopoietic Factors (Chugai), M. Sakaki in Division of Stem Cell Regulation (Amgen), Donation Laboratories; Prof. Yoshida, Y. Nakatake, T. Shiota in Laboratory of Gene Expression and Regulation, K. Kagiwada in Laboratory of Cell Biology, Center for Experimental Medicine; M. Handa, E. Suzuki and M. Sato in Inspection room, Prof. Tani,

M. Kasui, H. Yokokawa and S. Suzuki in Division of Molecular Therapy,The Advanced Clinical Research Center and K. Y. Kamata in Coop for many advices and encouragements.

All animals for my studies for their lives. Particularly, smart beagle sisters: Pinokio, Bobby, Michael, Bull, Mask, Shack, Boss, Black, Chappy, Chibi and Boo.

References

Adams, B., Chan, A., Callahan, H. & Milgram, N. W. (2000). The canine as a model of human cognitive aging: recent developments. Prog Neuropsychopharmacol Biol Psychiatry 24, 675-92.

Aguzzi, A., Brandner, S., Sure, U., Ruedi, D. & Isenmann, S. (1994). Transgenic and knock-out mice: models of neurological disease. Brain Pathol 4, 3-20.

Andersson, T., Schwarcz, R., Love, A. & Kristensson, K. (1993). Measles virus-induced hippocampal neurodegeneration in the mouse: a novel, subacute model for testing neuroprotective agents. Neurosci Lett 154, 109-12.

Appel, M. J., Glickman, L. T., Raine, C. S. & Tourtellotte, W. W. (1981). Canine viruses and multiple sclerosis. Neurology 31, 944-9.

Appel, M. J., Yates, R. A., Foley, G. L., Bernstein, J. J., Santinelli, S., Spelman, L. H., Miller, L. D., Arp, L. H., Anderson, M., Barr, M. & et al. (1994). Canine distemper epizootic in lions, tigers, and leopards in North America. J Vet Diagn Invest 6, 277-88.

Asanuma, M., Hirata, H. & Cadet, J. L. (1998). Attenuation of 6-hydroxydopamine-induced dopaminergic nigrostriatal lesions in superoxide dismutase transgenic mice. Neuroscience 85, 907-17.

Baczko, K., Liebert, U. G., Billeter, M., Cattaneo, R., Budka, H. & ter Meulen, V. (1986). Expression of defective measles virus genes in brain tissues of patients with subacute sclerosing panencephalitis. J Virol 59, 472-8.

Barendse, W., Armitage, S. M., Ryan, A. M., Moore, S. S., Clayton, D., Georges, M., Womack, J. E. & Hetzel, J. (1993). A genetic map of DNA loci on bovine chromosome 1. Genomics 18, 602-8.

Barkats, M., Nakao, N., Grasbon-Frodl, E. M., Bilang-Bleuel, A., Revah, F., Mallet, J. & Brundin, P. (1997). Intrastriatal grafts of embryonic mesencephalic rat neurons genetically modified using an adenovirus encoding human Cu/Zn superoxide dismutase. Neuroscience 78, 703-13.

Barkats, M., Millecamps, S., Bilang-Bleuel, A. & Mallet, J. (2002). Neuronal transfer of

the human Cu/Zn superoxide dismutase gene increases the resistance of dopaminergic neurons to 6-hydroxydopamine. J Neurochem 82, 101-9.

Baron, M. D. & Barrett, T. (1997). Rescue of rinderpest virus from cloned cDNA. J Virol 71, 1265-71.

Beauchamp, C. & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44, 276-87.

Bestetti, G., Fatzer, R. & Frankhauser, R. (1978). Encephalitis following vaccination against distemper and infectious hepatitis in the dog. An optical and ultrastructural study. Acta Neuropathol (Berl) 43, 69-75.

Blixenkrone-Moller, M., Svansson, V., Appel, M., Krogsrud, J., Have, P. & Orvell, C. (1992). Antigenic relationships between field isolates of morbilliviruses from different carnivores. Arch Virol 123, 279-94.

Bolt, G., Jensen, T. D., Gottschalck, E., Arctander, P., Appel, M. J., Buckland, R. & Blixenkrone-Moller, M. (1997). Genetic diversity of the attachment (H) protein gene of current field isolates of canine distemper virus. J Gen Virol 78 (Pt 2), 367-72.

Buchholz, U. J., Finke, S. & Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J Virol 73, 251-9.

Campos-Neto, A., Porrozzi, R., Greeson, K., Coler, R. N., Webb, J. R., Seiky, Y. A., Reed, S. G. & Grimaldi, G., Jr. (2001). Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect Immun 69, 4103-8.

Campos-Neto, A., Webb, J. R., Greeson, K., Coler, R. N., Skeiky, Y. A. & Reed, S. G. (2002). Vaccination with plasmid DNA encoding TSA/LmSTI1 leishmanial fusion proteins confers protection against Leishmania major infection in susceptible BALB/c mice. Infect Immun 70, 2828-36.

Castro, A. E., Burnstein, T. & Byington, D. P. (1972). Properties in cell cultures of a hamster brain-adapted subacute sclerosing panencephalitis virus. J Gen Virol 16, 413-7.

Cathomen, T., Mrkic, B., Spehner, D., Drillien, R., Naef, R., Pavlovic, J., Aguzzi, A., Billeter, M. A. & Cattaneo, R. (1998). A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain. Embo J 17, 3899-908.

Cattaneo, R., Schmid, A., Billeter, M. A., Sheppard, R. D. & Udem, S. A. (1988). Multiple viral mutations rather than host factors cause defective measles virus gene expression in a subacute sclerosing panencephalitis cell line. J Virol 62, 1388-97.

Chang, L. Y., Slot, J. W., Geuze, H. J. & Crapo, J. D. (1988). Molecular immunocytochemistry of the CuZn superoxide dismutase in rat hepatocytes. J Cell Biol 107, 2169-79.

Clarke, D. K., Sidhu, M. S., Johnson, J. E. & Udem, S. A. (2000). Rescue of mumps virus from cDNA. J Virol 74, 4831-8.

Coler, R. N., Skeiky, Y. A., Bernards, K., Greeson, K., Carter, D., Cornellison, C. D., Modabber, F., Campos-Neto, A. & Reed, S. G. (2002). Immunization with a polyprotein vaccine consisting of the T-Cell antigens thiol-specific antioxidant, Leishmania major stress-inducible protein 1, and Leishmania elongation initiation factor protects against leishmaniasis. Infect Immun 70, 4215-25.

Collins, P. L., Hill, M. G., Camargo, E., Grosfeld, H., Chanock, R. M. & Murphy, B. R. (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc Natl Acad Sci U S A 92, 11563-7.

Coltman, B. W. & Ide, C. F. (1996). Temporal characterization of microglia, IL-1 beta-like immunoreactivity and astrocytes in the dentate gyrus of hippocampal organotypic slice cultures. Int J Dev Neurosci 14, 707-19.

Cork, L. C., Adams, R. J., Griffin, J. W. & Price, D. L. (1982). Hereditary canine spinal

muscular atrophy: a canine model of human motor neuron disease. Prog Clin Biol Res 94, 449-58.

Cotman, C. W., E. Head, B. A. Muggenburg, S. Zicker, and N. W. Milgram. (2002) Brain aging in the canine: a diet enriched in antioxidants reduces cognitive dysfunction. Neurobiol, Aging 23:809-18

Crapo, J. D., Oury, T., Rabouille, C., Slot, J. W. & Chang, L. Y. (1992). Copper,zinc superoxide dismutase is primarily a cytosolic protein in human cells. Proc Natl Acad Sci U S A 89, 10405-9.

Creel, S., Creel, N. M., Munson, L., Sanderlin, D. & Appel, M. J. (1997). Serosurvey for selected viral diseases and demography of African wild dogs in Tanzania. J Wildl Dis 33, 823-32.

Cummings, B. J., Su, J. H., Cotman, C. W., White, R. & Russell, M. J. (1993). Beta-amyloid accumulation in aged canine brain: a model of early plaque formation in Alzheimer's disease. Neurobiol Aging 14, 547-60.

Cummings, B. J., Head, E., Afagh, A. J., Milgram, N. W. & Cotman, C. W. (1996). Beta-amyloid accumulation correlates with cognitive dysfunction in the aged canine. Neurobiol Learn Mem 66, 11-23.

Curran, J., Marq, J. B. & Kolakofsky, D. (1995). An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J Virol 69, 849-55.

Curran, J., Boeck, R., Lin-Marq, N., Lupas, A. & Kolakofsky, D. (1995). Paramyxovirus phosphoproteins form homotrimers as determined by an epitope dilution assay, via predicted coiled coils. Virology 214, 139-49.

Curran, J. (1996). Reexamination of the Sendai virus P protein domains required for RNA synthesis: a possible supplemental role for the P protein. Virology 221, 130-40.

Cypher, B. L., Scrivner, J. H., Hammer, K. L. & O'Farrell, T. P. (1998). Viral antibodies in coyotes from California. J Wildl Dis 34, 259-64.

Dahlberg, J. E. & Simon, E. H. (1969). Physical and genetic studies of Newcastle disease virus: evidence for multiploid particles. Virology 38, 666-78.

Das, T., Schuster, A., Schneider-Schaulies, S. & Banerjee, A. K. (1995). Involvement of cellular casein kinase II in the phosphorylation of measles virus P protein: identification of phosphorylation sites. Virology 211, 218-26.

Das, T., Pattnaik, A. K., Takacs, A. M., Li, T., Hwang, L. N. & Banerjee, A. K. (1997). Basic amino acid residues at the carboxy-terminal eleven amino acid region of the phosphoprotein (P) are required for transcription but not for replication of vesicular stomatitis virus genome RNA. Virology 238, 103-14.

Das, T., Chakrabarti, B. K., Chattopadhyay, D. & Banerjee, A. K. (1999). Carboxy-terminal five amino acids of the nucleocapsid protein of vesicular stomatitis virus are required for encapsidation and replication of genome RNA. Virology 259, 219-27.

Das, S. C., Baron, M. D. & Barrett, T. (2000). Recovery and characterization of a chimeric rinderpest virus with the glycoproteins of peste-des-petits-ruminants virus: homologous F and H proteins are required for virus viability. J Virol 74, 9039-47.

Davis, J. M., Rosenfeld, W. N., Richter, S. E., Parad, M. R., Gewolb, I. H., Spitzer, A. R., Carlo, W. A., Couser, R. J., Price, A., Flaster, E., Kassem, N., Edwards, L., Tierney, J. & Horowitz, S. (1997). Safety and pharmacokinetics of multiple doses of recombinant human CuZn superoxide dismutase administered intratracheally to premature neonates with respiratory distress syndrome. Pediatrics 100, 24-30.

De, B. P., Hoffman, M. A., Choudhary, S., Huntley, C. C. & Banerjee, A. K. (2000). Role of NH(2)- and COOH-terminal domains of the P protein of human parainfluenza virus type 3 in transcription and replication. J Virol 74, 5886-95.

Dietze, R., Barros, G. B., Teixeira, L., Harris, J., Michelson, K., Falqueto, A. & Corey, R. (1997). Effect of eliminating seropositive canines on the transmission of visceral leishmaniasis in Brazil. Clin Infect Dis 25, 1240-2.

Dubois-Dalcq, M., Reese, T. S., Murphy, M. & Fuccillo, D. (1976). Defective bud formation in human cells chronically infected with subacute sclerosing panencephalitis virus. J Virol 19, 579-93.

Dunbar, M. R., Cunningham, M. W. & Roof, J. C. (1998). Seroprevalence of selected disease agents from free-ranging black bears in Florida. J Wildl Dis 34, 612-9.

Duprex, W. P., Duffy, I., McQuaid, S., Hamill, L., Cosby, S. L., Billeter, M. A., Schneider-Schaulies, J., ter Meulen, V. & Rima, B. K. (1999). The H gene of rodent brain-adapted measles virus confers neurovirulence to the Edmonston vaccine strain. J Virol 73, 6916-22.

Durbin, A. P., Hall, S. L., Siew, J. W., Whitehead, S. S., Collins, P. L. & Murphy, B. R. (1997). Recovery of infectious human parainfluenza virus type 3 from cDNA. Virology 235, 323-32.

Ek-Kommonen, C., Sihvonen, L., Pekkanen, K., Rikula, U. & Nuotio, L. (1997). Outbreak off canine distemper in vaccinated dogs in Finland. Vet Rec 141, 380-3.

Emerson, (1987) , Plenum, New York pp. 245-269

Estevez, A. G., Sampson, J. B., Zhuang, Y. X., Spear, N., Richardson, G. J., Crow, J. P., Tarpey, M. M., Barbeito, L. & Beckman, J. S. (2000). Liposome-delivered superoxide dismutase prevents nitric oxide-dependent motor neuron death induced by trophic factor withdrawal. Free Radic Biol Med 28, 437-46.

Evlashev, A., Moyse, E., Valentin, H., Azocar, O., Trescol-Biemont, M. C., Marie, J. C., Rabourdin-Combe, C. & Horvat, B. (2000). Productive measles virus brain infection and apoptosis in CD46 transgenic mice. J Virol 74, 1373-82.

Fankhauser, R. (1982). [Canine distemper--the history of a disease]. Schweiz Arch Tierheilkd 124, 245-56.

Fields Virology, Forth Edition, 2001, Lippincott Williams & Wilkins

Fooks, A. R., Stephenson, J. R., Warnes, A., Dowsett, A. B., Rima, B. K. & Wilkinson, G.

W. (1993). Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. J Gen Virol 74 (Pt 7), 1439-44.

Fooks, A. R., Schadeck, E., Liebert, U. G., Dowsett, A. B., Rima, B. K., Steward, M., Stephenson, J. R. & Wilkinson, G. W. (1995). High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: induction of an MHC-1-restricted CTL response and protection in a murine model. Virology 210, 456-65.

Forsyth, M. A., Kennedy, S., Wilson, S., Eybatov, T. & Barrett, T. (1998). Canine distemper virus in a Caspian seal. Vet Rec 143, 662-4.

Frauchiger, E. (1968). [Demyelination diseases in the animal]. Bull Schweiz Akad Med Wiss 24, 66-72.

Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J. & Kolakofsky, D. (1995). A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. Embo J 14, 6087-94.

Gassen, U., Collins, F. M., Duprex, W. P. & Rima, B. K. (2000). Establishment of a rescue system for canine distemper virus. J Virol 74, 10737-44.

Gemma, T., Iwatsuki, K., Shin, Y. S., Yoshida, E., Kai, C. & Mikami, T. (1996). Serological analysis of canine distemper virus using an immunocapture ELISA. J Vet Med Sci 58, 791-4.

Gemma, T., Watari, T., Akiyama, K., Miyashita, N., Shin, Y. S., Iwatsuki, K., Kai, C. & Mikami, T. (1996). Epidemiological observations on recent outbreaks of canine distemper in Tokyo area. J Vet Med Sci 58, 547-50.

Green, S. L., Tolwani, R. J., Varma, S., Quignon, P., Galibert, F. & Cork, L. C. (2002). Structure, chromosomal location, and analysis of the canine Cu/Zn superoxide dismutase (SOD1) gene. J Hered 93, 119-24.

Groner, Y., Avraham, K. B., Schickler, M., Yarom, R. & Knobler, H. (1990). Clinical

symptoms of Down syndrome are manifested in transgenic mice overexpressing the human Cu/Zn-superoxide dismutase gene. Prog Clin Biol Res 360, 233-62.

Groner, Y. (1995). Transgenic models for chromosome 21 gene dosage effects. Prog Clin Biol Res 393, 193-212.

Gurunathan, S., Prussin, C., Sacks, D. L. & Seder, R. A. (1998). Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nat Med 4, 1409-15.

Haas, L., Hofer, H., East, M., Wohlsein, P., Liess, B. & Barrett, T. (1996). Canine distemper virus infection in Serengeti spotted hyenas. Vet Microbiol 49, 147-52.

Haas, L., Martens, W., Greiser-Wilke, I., Mamaev, L., Butina, T., Maack, D. & Barrett, T. (1997). Analysis of the haemagglutinin gene of current wild-type canine distemper virus isolates from Germany. Virus Res 48, 165-71.

Haile, R., Smith, P., Read, D., Nassim, D., Warlow, C. & Russell, W. C. (1982). A study of measles virus and canine distemper virus antibodies, and of childhood infections in multiple sclerosis patients and controls. J Neurol Sci 56, 1-10.

Haller, A. A., Miller, T., Mitiku, M. & Coelingh, K. (2000). Expression of the surface glycoproteins of human parainfluenza virus type 3 by bovine parainfluenza virus type 3, a novel attenuated virus vaccine vector. J Virol 74, 11626-35.

Harder, T. C., Kenter, M., Vos, H., Siebelink, K., Huisman, W., van Amerongen, G., Orvell, C., Barrett, T., Appel, M. J. & Osterhaus, A. D. (1996). Canine distemper virus from diseased large felids: biological properties and phylogenetic relationships. J Gen Virol 77 (Pt 3), 397-405.

Harty, R. N. & Palese, P. (1995). Measles virus phosphoprotein (P) requires the NH2- and COOH-terminal domains for interactions with the nucleoprotein (N) but only the COOH terminus for interactions with itself. J Gen Virol 76 (Pt 11), 2863-7.

Harvey, F. H. (1975). The large dense core vesicle: a normal organelle of the central nervous system myelinated axon. Acta Neuropathol (Berl) 31, 91-6.

He, B., Paterson, R. G., Ward, C. D. & Lamb, R. A. (1997). Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. Virology 237, 249-60.

Head, E., Garzon-Rodriguez, W., Johnson, J. K., Lott, I. T., Cotman, C. W. & Glabe, C. (2001). Oxidation of Abeta and plaque biogenesis in Alzheimer's disease and Down syndrome. Neurobiol Dis 8, 792-806.

Ho, Y. S., Gargano, M., Cao, J., Bronson, R. T., Heimler, I. & Hutz, R. J. (1998). Reduced fertility in female mice lacking copper-zinc superoxide dismutase. J Biol Chem 273, 7765-9.

Hoffman, M. A. & Banerjee, A. K. (1997). An infectious clone of human parainfluenza virus type 3. J Virol 71, 4272-7.

Horikami, S. M., Curran, J., Kolakofsky, D. & Moyer, S. A. (1992). Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. J Virol 66, 4901-8.

Hosaka, Y., Kitano, H. & Ikeguchi, S. (1966). Studies on the pleomorphism of HVJ virons. Virology 29, 205-21.

Huber, M., Cattaneo, R., Spielhofer, P., Orvell, C., Norrby, E., Messerli, M., Perriard, J. C. & Billeter, M. A. (1991). Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. Virology 185, 299-308.

Hur, K., Bae, J. S., Choi, J. H., Kim, J. H., Kwon, S. W., Lee, K. W. & Kim, D. Y. (1999). Canine distemper virus infection in binturongs (Arctictis binturong). J Comp Pathol 121, 295-9.

Imaizumi, S., Woolworth, V., Fishman, R. A. & Chan, P. H. (1990). Liposome-entrapped superoxide dismutase reduces cerebral infarction in cerebral ischemia in rats. Stroke 21, 1312-7.

Innes, J. R. & Shoho, C. (1952). Nematodes, nervous disease, and neurotropic virus infection; observations in animal pathology of probable significance in medical

neurology. Br Med J 2, 366-8.

Iwatsuki, K., Okita, M., Ochikubo, F., Gemma, T., Shin, Y. S., Miyashita, N., Mikami, T. & Kai, C. (1995). Immunohistochemical analysis of the lymphoid organs of dogs naturally infected with canine distemper virus. J Comp Pathol 113, 185-90.

Iwatsuki, K., Miyashita, N., Yoshida, E., Gemma, T., Shin, Y. S., Mori, T., Hirayama, N., Kai, C. & Mikami, T. (1997). Molecular and phylogenetic analyses of the haemagglutinin (H) proteins of field isolates of canine distemper virus from naturally infected dogs. J Gen Virol 78 (Pt 2), 373-80.

Iwatsuki, K., Miyashita, N., Yoshida, E., Shin, Y. S., Ohashi, K., Kai, C. & Mikami, T. (1998). The nucleotide and predicted amino acid sequence of the fusion protein of recent isolates of canine distemper virus in Japan. J Vet Med Sci 60, 381-5.

Iwatsuki, K., Tokiyoshi, S., Hirayama, N., Nakamura, K., Ohashi, K., Wakasa, C., Mikami, T. & Kai, C. (2000). Antigenic differences in the H proteins of canine distemper viruses. Vet Microbiol 71, 281-6.

Johansson, K., Bourhis, J. M., Campanacci, V., Cambillau, C., Canard, B. & Longhi, S. (2003). Crystal structure of the measles virus phosphoprotein domain responsible for the induced folding of the C-terminal domain of the nucleoprotein. J Biol Chem 278, 44567-73.

Jozwik, A. & Frymus, T. (2002). Natural distemper in vaccinated and unvaccinated dogs in Warsaw. J Vet Med B Infect Dis Vet Public Health 49, 413-4.

Kai, C., Ochikubo, F., Okita, M., Iinuma, T., Mikami, T., Kobune, F. & Yamanouchi, K. (1993). Use of B95a cells for isolation of canine distemper virus from clinical cases. J Vet Med Sci 55, 1067-70.

Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M. & Nagai, Y. (1996). Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. Genes Cells 1, 569-79.

Kawamoto, K., Sha, S. H., Minoda, R., Izumikawa, M., Kuriyama, H., Schacht, J. &

Raphael, Y. (2004). Antioxidant gene therapy can protect hearing and hair cells from ototoxicity. Mol Ther 9, 173-81.

Kawana-Tachikawa, A., Tomizawa, M., Nunoya, J., Shioda, T., Kato, A., Nakayama, E. E., Nakamura, T., Nagai, Y. & Iwamoto, A. (2002). An efficient and versatile mammalian viral vector system for major histocompatibility complex class I/peptide complexes. J Virol 76, 11982-8.

Kawano, M., Kaito, M., Kozuka, Y., Komada, H., Noda, N., Nanba, K., Tsurudome, M., Ito, M., Nishio, M. & Ito, Y. (2001). Recovery of infectious human parainfluenza type 2 virus from cDNA clones and properties of the defective virus without V-specific cysteine-rich domain. Virology 284, 99-112.

Keller, G. A., Warner, T. G., Steimer, K. S. & Hallewell, R. A. (1991). Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. Proc Natl Acad Sci U S A 88, 7381-5.

Kim, C. D., Shin, H. K., Lee, H. S., Lee, J. H., Lee, T. H. & Hong, K. W. (2002). Gene transfer of Cu/Zn SOD to cerebral vessels prevents FPI-induced CBF autoregulatory dysfunction. Am J Physiol Heart Circ Physiol 282, H1836-42.

Kingston, R. L., Hamel, D. J., Gay, L. S., Dahlquist, F. W. & Matthews, B. W. (2004). Structural basis for the attachment of a paramyxoviral polymerase to its template. Proc Natl Acad Sci U S A 101, 8301-6.

Knobil, K., Choi, A. M., Weigand, G. W. & Jacoby, D. B. (1998). Role of oxidants in influenza virus-induced gene expression. Am J Physiol 274, L134-42.

Kobune, K., Kobune, F., Yamanouchi, K., Nagashima, K., Yoshikawa, Y. & Hayami, M. (1983). Neurovirulence of rat brain-adapted measles virus. Jpn J Exp Med 53, 177-80.

Kobune, F., Sakata, H. & Sugiura, A. (1990). Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. J Virol 64, 700-5.

Kock, R., Chalmers, W. S., Mwanzia, J., Chillingworth, C., Wambua, J., Coleman, P. G. & Baxendale, W. (1998). Canine distemper antibodies in lions of the Masai Mara. Vet Rec

142, 662-5.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. & Roux, L. (1998). Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. J Virol 72, 891-9.

Krakowka, S., Mador, R. A. & Koestner, A. (1978). Canine distemper virus-associated encephalitis: modification by passive antibody administration. Acta Neuropathol (Berl) 43, 235-41.

Krishnamurthy, S., Huang, Z. & Samal, S. K. (2000). Recovery of a virulent strain of newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. Virology 278, 168-82.

Launois, P., Maillard, I., Pingel, S., Swihart, K. G., Xenarios, I., Acha-Orbea, H., Diggelmann, H., Locksley, R. M., MacDonald, H. R. & Louis, J. A. (1997). IL-4 rapidly produced by V beta 4 V alpha 8 CD4+ T cells instructs Th2 development and susceptibility to Leishmania major in BALB/c mice. Immunity 6, 541-9.

Lawrence, D. M., Patterson, C. E., Gales, T. L., D'Orazio, J. L., Vaughn, M. M. & Rall, G. F. (2000). Measles virus spread between neurons requires cell contact but not CD46 expression, syncytium formation, or extracellular virus production. J Virol 74, 1908-18.

Lawson, N. D., Stillman, E. A., Whitt, M. A. & Rose, J. K. (1995). Recombinant vesicular stomatitis viruses from DNA. Proc Natl Acad Sci U S A 92, 4477-81.

Likhoshway Ye, V., Grachev, M. A., Kumarev, V. P., Solodun Yu, V., Goldberg, O. A., Belykh, O. I., Nagieva, F. G., Nikulina, V. G. & Kolesnik, B. S. (1989). Baikal seal virus. Nature 339, 266.

Lincoln, S. D., Gorham, J. R., Ott, R. L. & Hegreberg, G. A. (1971). Etiologic studies of old dog encephalitis. I. Demonstration of canine distemper viral antigen in the brain in two cases. Vet Pathol 8, 1-8.

Lincoln, S. D., Gorham, J. R., Davis, W. C. & Ott, R. L. (1973). Studies of old dog encephalitis. II. Electron microscopic and immunohistologic findings. Vet Pathol 10,

124-9.

Liou, W., Chang, L. Y., Geuze, H. J., Strous, G. J., Crapo, J. D. & Slot, J. W. (1993). Distribution of CuZn superoxide dismutase in rat liver. Free Radic Biol Med 14, 201-7.

Lisiak, J. A. & Vandevelde, M. (1979). Polioencephalomalacia associated with canine distemper virus infection. Vet Pathol 16, 650-60.

Liston, P. & Briedis, D. J. (1995). Ribosomal frameshifting during translation of measles virus P protein mRNA is capable of directing synthesis of a unique protein. J Virol 69, 6742-50.

Longhi, S., Receveur-Brechot, V., Karlin, D., Johansson, K., Darbon, H., Bhella, D., Yeo, R., Finet, S. & Canard, B. (2003). The C-terminal domain of the measles virus nucleoprotein is intrinsically disordered and folds upon binding to the C-terminal moiety of the phosphoprotein. J Biol Chem 278, 18638-48.

Lorin, C., Mollet, L., Delebecque, F., Combredet, C., Hurtrel, B., Charneau, P., Brahic, M. & Tangy, F. (2004). A single injection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. J Virol 78, 146-57.

Lund, G. A., Tyrrell, D. L., Bradley, R. D. & Scraba, D. G. (1984). The molecular length of measles virus RNA and the structural organization of measles nucleocapsids. J Gen Virol 65 (Pt 9), 1535-42.

Mamaev, L. V., Denikina, N. N., Belikov, S. I., Volchkov, V. E., Visser, I. K., Fleming, M., Kai, C., Harder, T. C., Liess, B., Osterhaus, A. D. & et al. (1995). Characterisation of morbilliviruses isolated from Lake Baikal seals (Phoca sibirica). Vet Microbiol 44, 251-9.

Mamaev, L. V., Visser, I. K., Belikov, S. I., Denikina, N. N., Harder, T., Goatley, L., Rima, B., Edginton, B., Osterhaus, A. D. & Barrett, T. (1996). Canine distemper virus in Lake Baikal seals (Phoca sibirica). Vet Rec 138, 437-9.

Marsden, P. D. (1984). Selective primary health care: strategies for control of disease in the developing world. XIV. Leishmaniasis. Rev Infect Dis 6, 736-44.

Marsilio, F., Tiscar, P. G., Gentile, L., Roth, H. U., Boscagli, G., Tempesta, M. & Gatti, A. (1997). Serologic survey for selected viral pathogens in brown bears from Italy. J Wildl Dis 33, 304-7.

McCullough, B., Krakowka, S. & Koestner, A. (1974). Experimental canine distemper virus-induced demyelination. Lab Invest 31, 216-22.

McQuaid, S., Campbell, S., Wallace, I. J., Kirk, J. & Cosby, S. L. (1998). Measles virus infection and replication in undifferentiated and differentiated human neuronal cells in culture. J Virol 72, 5245-50.

Mellon, M. G. & Emerson, S. U. (1978). Rebinding of transcriptase components (L and NS proteins) to the nucleocapsid template of vesicular stomatitis virus. J Virol 27, 560-7.

Mendez, S., Gurunathan, S., Kamhawi, S., Belkaid, Y., Moga, M. A., Skeiky, Y. A., Campos-Neto, A., Reed, S., Seder, R. A. & Sacks, D. (2001). The potency and durability of DNA- and protein-based vaccines against Leishmania major evaluated using low-dose, intradermal challenge. J Immunol 166, 5122-8.

Mitra-Kaushik, S., Nayak, R. & Shaila, M. S. (2001). Identification of a cytotoxic T-cell epitope on the recombinant nucleocapsid proteins of Rinderpest and Peste des petits ruminants viruses presented as assembled nucleocapsids. Virology 279, 210-20.

R Miura, A Takenaka, M Ejima, M Yoneda, Y Goto, C Sanjoba, Y Matsumoto, Y Endo K Kohara and C Kai, July 20-24 (2002), "DEVELOPMENT OF A POLYVALENT CDV VACCINE AGAINST CDV AND *LEISHMANIA* INFECTION." 21st Annual Meeting of American Society for Virology: W29-3

Miyagawa, S., Ikawa, M., Kominami, K., Tanaka, H., Mikata, S., Matsuda, H., Seya, T., Shirakura, R. & Okabe, M. (1997). The regulation of membrane cofactor protein (CD46) expression in transgenic mice: the importance of the first 125 BP of the 3' untranslated region. Transplant Proc 29, 941-2.

Morell, V. (1996). New virus variant killed Serengeti cats. Science 271, 596.

Mougneau, E., Altare, F., Wakil, A. E., Zheng, S., Coppola, T., Wang, Z. E., Waldmann, R., Locksley, R. M. & Glaichenhaus, N. (1995). Expression cloning of a protective Leishmania antigen. Science 268, 563-6.

Naim, H. Y., Ehler, E. & Billeter, M. A. (2000). Measles virus matrix protein specifies apical virus release and glycoprotein sorting in epithelial cells. Embo J 19, 3576-85.

Nakai, M. & Imagawa, D. T. (1969). Electron microscopy of measles virus replication. J Virol 3, 187-97.

Nakao, N., Frodl, E. M., Widner, H., Carlson, E., Eggerding, F. A., Epstein, C. J. & Brundin, P. (1995). Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. Nat Med 1, 226-31.

Narang, H. K. (1982). Ultrastructural study of long-term canine distemper virus infection in tissue culture cells. Infect Immun 36, 310-9.

Neumann, G., Feldmann, H., Watanabe, S., Lukashevich, I. & Kawaoka, Y. (2002). Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J Virol 76, 406-10.

Oberley, L. W. & Buettner, G. R. (1979). Role of superoxide dismutase in cancer: a review. Cancer Res 39, 1141-9.

Ohashi, K., Miyazaki, N., Tanabe, S., Nakata, H., Miura, R., Fujita, K., Wakasa, C., Uema, M., Shiotani, M., Takahashi, E. & Kai, C. (2001). Seroepidemiological survey of distemper virus infection in the Caspian Sea and in Lake Baikal. Vet Microbiol 82, 203-10.

Ohishi, K., Inui, K., Yamanouchi, K. & Barrett, T. (1999). Cell-mediated immune responses in cattle vaccinated with a vaccinia virus recombinant expressing the nucleocapsid protein of rinderpest virus. J Gen Virol 80 (Pt 7), 1627-34.

Okita, M., Yanai, T., Ochikubo, F., Gemma, T., Mori, T., Maseki, T., Yamanouchi, K., Mikami, T. & Kai, C. (1997). Histopathological features of canine distemper recently observed in Japan. J Comp Pathol 116, 403-8.

Okuno, T., Takeuchi, M., Matsumoto, Y. & Otsuka, H. (2002). Pretreatment of leishmania homologue of receptors for activated C kinase (LACK) promotes disease progression caused by Leishmania amazonensis. Exp Anim 51, 335-41.

Oldstone, M. B., Lewicki, H., Thomas, D., Tishon, A., Dales, S., Patterson, J., Manchester, M., Homann, D., Naniche, D. & Holz, A. (1999). Measles virus infection in a transgenic model: virus-induced immunosuppression and central nervous system disease. Cell 98, 629-40.

Orvell, C., Sheshberadaran, H. & Norrby, E. (1985). Preparation and characterization of monoclonal antibodies directed against four structural components of canine distemper virus. J Gen Virol 66 (Pt 3), 443-56.

Osterhaus, A. (1989). A morbillivirus causing mass mortality in seals. Vaccine 7, 483-4.

Osterhaus, A. D., Broeders, H. W., Groen, J., UytdeHaag, F. G., Visser, I. K., van de Bildt, M. W., Orvell, C., Kumarev, V. P. & Zorin, V. L. (1989). Different morbilliviruses in European and Siberian seals. Vet Rec 125, 647-8.

Osterhaus, A. D., Groen, J., UytdeHaag, F. G., Visser, I. K., Vedder, E. J., Crowther, J. & Bostock, C. J. (1989). Morbillivirus infections in European seals before 1988. Vet Rec 125, 326.

Oyanagi, S., ter Meulen, V., Katz, M. & Koprowski, H. (1971). Comparison of subacute sclerosing panencephalitis and measles viruses: an electron microscope study. J Virol 7, 176-87.

Palese, P. (1995). Genetic engineering of infectious negative-strand RNA viruses. Trends Microbiol 3, 123-5.

Palosaari, H., Parisien, J. P., Rodriguez, J. J., Ulane, C. M. & Horvath, C. M. (2003). STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. J Virol 77, 7635-44.

Papaioannou, N., Tooten, P. C., van Ederen, A. M., Bohl, J. R., Rofina, J., Tsangaris, T. &

Gruys, E. (2001). Immunohistochemical investigation of the brain of aged dogs. I. Detection of neurofibrillary tangles and of 4-hydroxynonenal protein, an oxidative damage product, in senile plaques. Amyloid 8, 11-21.

Parks, C. L., Lerch, R. A., Walpita, P., Sidhu, M. S. & Udem, S. A. (1999). Enhanced measles virus cDNA rescue and gene expression after heat shock. J Virol 73, 3560-6.

Parks, C. L., Wang, H. P., Kovacs, G. R., Vasilakis, N., Kowalski, J., Nowak, R. M., Lerch, R. A., Walpita, P., Sidhu, M. S. & Udem, S. A. (2002). Expression of a foreign gene by recombinant canine distemper virus recovered from cloned DNAs. Virus Res 83, 131-47.

Peeters, B. P., de Leeuw, O. S., Koch, G. & Gielkens, A. L. (1999). Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J Virol 73, 5001-9.

Plattet, P., Zweifel, C., Wiederkehr, C., Belloy, L., Cherpillod, P., Zurbriggen, A. & Wittek, R. (2004). Recovery of a persistent Canine distemper virus expressing the enhanced green fluorescent protein from cloned cDNA. Virus Res 101, 147-53.

Plumb, J., Duprex, W. P., Cameron, C. H., Richter-Landsberg, C., Talbot, P. & McQuaid, S. (2002). Infection of human oligodendroglioma cells by a recombinant measles virus expressing enhanced green fluorescent protein. J Neurovirol 8, 24-34.

Poch, O., Blumberg, B. M., Bougueleret, L. & Tordo, N. (1990). Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J Gen Virol 71 (Pt 5), 1153-62.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G. & Billeter, M. A. (1995). Rescue of measles viruses from cloned DNA. Embo J 14, 5773-84.

Rager, M., Vongpunsawad, S., Duprex, W. P. & Cattaneo, R. (2002). Polyploid measles virus with hexameric genome length. Embo J 21, 2364-72.

Rall, G. F., Manchester, M., Daniels, L. R., Callahan, E. M., Belman, A. R. & Oldstone, M.

B. (1997). A transgenic mouse model for measles virus infection of the brain. Proc Natl Acad Sci U S A 94, 4659-63.

Ramiro, M. J., Zarate, J. J., Hanke, T., Rodriguez, D., Rodriguez, J. R., Esteban, M., Lucientes, J., Castillo, J. A. & Larraga, V. (2003). Protection in dogs against visceral leishmaniasis caused by Leishmania infantum is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. Vaccine 21, 2474-84.

Raoul, H., Mabondzo, A., Le Naour, R. & Dormont, D. (1994). Effect of human immunodeficiency virus type 1 (HIV-1) monocyte-derived macrophages infection on the manganous superoxide dismutase gene expression. Chem Biol Interact 91, 123-31.

Robbins, S. J. & Bussell, R. H. (1979). Structural phosphoproteins associated with purified measles virions and cytoplasmic nucleocapsids. Intervirology 12, 96-102.

Robert A. Lamb nad Daniel Kolakofsky, (2001), Paramyxoviridae: The viruses and their Replication, Fields Virology, Chapter 41, pp1305-1340

Roelke-Parker, M. E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S. J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H. & et al. (1996). A canine distemper virus epidemic in Serengeti lions (Panthera leo). Nature 379, 441-5.

Romer-Oberdorfer, A., Mundt, E., Mebatsion, T., Buchholz, U. J. & Mettenleiter, T. C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. J Gen Virol 80 (Pt 11), 2987-95.

Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H. X. & et al. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59-62.

Ruehl, W. W., Bruyette, D. S., DePaoli, A., Cotman, C. W., Head, E., Milgram, N. W. & Cummings, B. J. (1995). Canine cognitive dysfunction as a model for human age-related cognitive decline, dementia and Alzheimer's disease: clinical presentation, cognitive testing, pathology and response to 1-deprenyl therapy. Prog Brain Res 106,

217-25.

Sacks, D. & Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol 2, 845-58.

Sagi, A., Ferder, M., Levens, D. & Strauch, B. (1986). Improved survival of island flaps after prolonged ischemia by perfusion with superoxide dismutase. Plast Reconstr Surg 77, 639-44.

Saunders, L. Z. (1973). Some historical aspects of the neuropathology of canine distemper. Schweiz Arch Neurol Neurochir Psychiatr 112, 341-52.

Schneider, H., Spielhofer, P., Kaelin, K., Dotsch, C., Radecke, F., Sutter, G. & Billeter, M. A. (1997). Rescue of measles virus using a replication-deficient vaccinia-T7 vector. J Virol Methods 64, 57-64.

Schneider-Schaulies, S., Schneider-Schaulies, J., Schuster, A., Bayer, M., Pavlovic, J. & ter Meulen, V. (1994). Cell type-specific MxA-mediated inhibition of measles virus transcription in human brain cells. J Virol 68, 6910-7.

Schnell, M. J., Mebatsion, T. & Conzelmann, K. K. (1994). Infectious rabies viruses from cloned cDNA. Embo J 13, 4195-203.

Schobesberger, M., Zurbriggen, A., Doherr, M. G., Weissenbock, H., Vandevelde, M., Lassmann, H. & Griot, C. (2002). Demyelination precedes oligodendrocyte loss in canine distemper virus-induced encephalitis. Acta Neuropathol (Berl) 103, 11-9.

Schoehn, G., Mavrakis, M., Albertini, A., Wade, R., Hoenger, A. & Ruigrok, R. W. (2004). The 12 A structure of trypsin-treated measles virus N-RNA. J Mol Biol 339, 301-12.

Semrau, F., Kuhl, R. J., Ritter, S. & Ritter, K. (1998). Manganese superoxide dismutase (MnSOD) and autoantibodies against MnSOD in acute viral infections. J Med Virol 55, 161-7.

Sha, S. H., Zajic, G., Epstein, C. J. & Schacht, J. (2001). Overexpression of copper/zinc-superoxide dismutase protects from kanamycin-induced hearing loss.

Audiol Neurootol 6, 117-23.

Sheshberadaran, H., Norrby, E., McCullough, K. C., Carpenter, W. C. & Orvell, C. (1986). The antigenic relationship between measles, canine distemper and rinderpest viruses studied with monoclonal antibodies. J Gen Virol 67 (Pt 7), 1381-92.

Skoumalova, A., Rofina, J., Schwippelova, Z., Gruys, E. & Wilhelm, J. (2003). The role of free radicals in canine counterpart of senile dementia of the Alzheimer type. Exp Gerontol 38, 711-9.

Skrzycki, M. & Czeczot, H. (2004). [Extracellular superoxide dismutase (EC-SOD)--structure, properties and functions]. Postepy Hig Med Dosw (Online) 58, 301-11.

Solas, A. B., Kalous, P., Davis, J. M. & Saugstad, O. D. (2003). Effects of recombinant human superoxide dismutase during reoxygenation with 21% or 100% oxygen after cerebral asphyxia in newborn piglets. J Matern Fetal Neonatal Med 14, 96-101.

Spehner, D., Drillien, R. & Howley, P. M. (1997). The assembly of the measles virus nucleoprotein into nucleocapsid-like particles is modulated by the phosphoprotein. Virology 232, 260-8.

Stobie, L., Gurunathan, S., Prussin, C., Sacks, D. L., Glaichenhaus, N., Wu, C. Y. & Seder, R. A. (2000). The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc Natl Acad Sci U S A 97, 8427-32.

Stoppini, L., Buchs, P. A. & Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37, 173-82.

Sugiyama, M., Minamoto, N., Kinjo, T., Hirayama, N., Sasaki, H., Yoshikawa, Y. & Yamanouchi, K. (1989). Characterization of monoclonal antibodies against four structural proteins of rinderpest virus. J Gen Virol 70 (Pt 10), 2605-13.

Summers, B. A., Greisen, H. A. & Appel, M. J. (1979). Early events in canine distemper demyelinating encephalomyelitis. Acta Neuropathol (Berl) 46, 1-10.

Summers, B. A., Greisen, H. A. & Appel, M. J. (1984). Canine distemper encephalomyelitis: variation with virus strain. J Comp Pathol 94, 65-75.

Takeda, M., Takeuchi, K., Miyajima, N., Kobune, F., Ami, Y., Nagata, N., Suzaki, Y., Nagai, Y. & Tashiro, M. (2000). Recovery of pathogenic measles virus from cloned cDNA. J Virol 74, 6643-7.

Takenaka et al. 2005, "Establishment of a complete vaccination with a recent isolate in Japan, the Yanaka strain, against canine distemper" (in preparation).

Takeuchi, K., Kadota, S. I., Takeda, M., Miyajima, N. & Nagata, K. (2003). Measles virus V protein blocks interferon (IFN)-alpha/beta but not IFN-gamma signaling by inhibiting STAT1 and STAT2 phosphorylation. FEBS Lett 545, 177-82.

Tarbouriech, N., Curran, J., Ruigrok, R. W. & Burmeister, W. P. (2000). Tetrameric coiled coil domain of Sendai virus phosphoprotein. Nat Struct Biol 7, 777-81.

Tarbouriech, N., Curran, J., Ebel, C., Ruigrok, R. W. & Burmeister, W. P. (2000). On the domain structure and the polymerization state of the sendai virus P protein. Virology 266, 99-109.

Taylor, M. J., Godfrey, E., Baczko, K., ter Meulen, V., Wild, T. F. & Rima, B. K. (1991). Identification of several different lineages of measles virus. J Gen Virol 72 (Pt 1), 83-8.

Terabe, M., Kuramochi, T., Ito, M., Hatabu, T., Sanjoba, C., Chang, K. P., Onodera, T. & Matsumoto, Y. (2000). CD4(+) cells are indispensable for ulcer development in murine cutaneous leishmaniasis. Infect Immun 68, 4574-7.

van Moll, P., Alldinger, S., Baumgartner, W. & Adami, M. (1995). Distemper in wild carnivores: an epidemiological, histological and immunocytochemical study. Vet Microbiol 44, 193-9.

Vandevelde, M., Kristensen, B., Braund, K. G., Greene, C. E., Swango, L. J. & Hoerlein, B. F. (1980). Chronic canine distemper virus encephalitis in mature dogs. Vet Pathol 17, 17-28.

Vanloubbeeck, Y. & Jones, D. E. (2004). The immunology of leishmania infection and the implications for vaccine development. Ann N Y Acad Sci 1026, 267-72.

Volchkov, V. E., Volchkova, V. A., Muhlberger, E., Kolesnikova, L. V., Weik, M., Dolnik, O. & Klenk, H. D. (2001). Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science 291, 1965-9.

von Messling, V., Milosevic, D. & Cattaneo, R. (2004). Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. Proc Natl Acad Sci U S A 101, 14216-21.

Walsh, E. P., Baron, M. D., Rennie, L. F., Monaghan, P., Anderson, J. & Barrett, T. (2000). Recombinant rinderpest vaccines expressing membrane-anchored proteins as genetic markers: evidence of exclusion of marker protein from the virus envelope. J Virol 74, 10165-75.

Wang, M., Howell, J. M., Libbey, J. E., Tainer, J. A. & Fujinami, R. S. (2003). Manganese superoxide dismutase induction during measles virus infection. J Med Virol 70, 470-4.

Waterson, A. P. (1965). The Significance of Viral Structure. Arch Gesamte Virusforsch 15, 275-300.

Webb, J. R., Kaufmann, D., Campos-Neto, A. & Reed, S. G. (1996). Molecular cloning of a novel protein antigen of Leishmania major that elicits a potent immune response in experimental murine leishmaniasis. J Immunol 157, 5034-41.

Webb, J. R., Campos-Neto, A., Ovendale, P. J., Martin, T. I., Stromberg, E. J., Badaro, R. & Reed, S. G. (1998). Human and murine immune responses to a novel Leishmania major recombinant protein encoded by members of a multicopy gene family. Infect Immun 66, 3279-89.

Whelan, S. P., Ball, L. A., Barr, J. N. & Wertz, G. T. (1995). Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc Natl Acad Sci U S A 92, 8388-92.

Yarom, R., Sherman, Y., Sagher, U., Peled, I. J., Wexler, M. R. & Gorodetsky, R. (1987). Elevated concentrations of elements and abnormalities of neuromuscular junctions in tongue muscles of Down's syndrome. J Neurol Sci 79, 315-26.

Yoneda, M., Miura, R., Barrett, T., Tsukiyama-Kohara, K. & Kai, C. (2004). Rinderpest virus phosphoprotein gene is a major determinant of species-specific pathogenicity. J Virol 78, 6676-81.

Yoshida, E., Iwatsuki, K., Miyashita, N., Gemma, T., Kai, C. & Mikami, T. (1998). Molecular analysis of the nucleocapsid protein of recent isolates of canine distemper virus in Japan. Vet Microbiol 59, 237-44.

Zelko, I. N., Mariani, T. J. & Folz, R. J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33, 337-49.

Zurbriggen, A., Graber, H. U., Wagner, A. & Vandevelde, M. (1995). Canine distemper virus persistence in the nervous system is associated with noncytolytic selective virus spread. J Virol 69, 1678-86.

ABSTRACT IN JAPANEASE

論文の内容の要旨

農学生命科学科　獣医学専攻
平成１３年度博士課程　入学
氏　　名　　郡山　　尚紀
指導教官　　甲斐　知恵子

論文題目

Application of recombinant CDVs as leishmania vaccine and delivery vector

（組換えイヌジステンパーウイルスのリーシュマニアワクチンおよびデリバリーベクターとしての応用）

ジステンパーはイヌにおける代表的なウイルス性伝染病で、呼吸器系、消化器系、中枢神経系で病変が診られる致死率の高い疾病として知られている。原因となるイヌジステンパーウイルス（CDV）は麻疹ウイルス、牛疫ウイルスなどと共に、パラミクソウイルス科（Paramyxoviridae）モービリウイルス属（Morbillivirus）に分類される。最近まで CDV が属する一本鎖マイナス鎖 RNA ウイルス（モノネガウイルス）では、ゲノム cDNA クローンから感染性ウイルスを作出することができなかった。しかし、1994 年に狂犬病ウイルスにおいて初めて感染性ウイルス作出系(リバースジェネティクス系)が開発され、著者らの研究グループも 1999 年に世界で初めて CDV リバースジェネティクス系の開発に成功した。リバースジェネティクス系により、ウイルス構成遺伝子の欠失や交換、変異の導入や外来遺伝子の挿入を行った組換えウイルスの作出が可能になった。その結果、性質の異なるウイルス株での遺伝子解析の比較だけではわからなかった、病原性の決定機序や特異的な宿主を決定する機構等のウイルス学上重要な問題の解明に大きく寄与することとなった。さらに、液性免疫だけではなく細胞性免疫を誘導する優れた特性を生かしたウイルスベクターの開発やその応用も盛んに進められている。

本研究ではまず、蛍光蛋白 EGFP (Enhanced green fluorescent protein)発現組換えCDV を用いて、イヌ海馬における CDV の感染動態の解析を行った（第１章)。次に CDV デリバリーベクターへの応用例として、CDV リバースジェネティクス系により抗酸化酵素 Superoxide dismutase 1(SOD1)発現組換えウイルスを作出し、その評価を行った（第２章)。そして、リーシュマニア感染症に対する２価ワクチンベクターとして、リーシュマニア抗原発現組換え CDV を作出し、イヌに対する感染実験を行いその有効性を検討した（第３章)。また、CDV の基礎的な研究として、CDV P蛋白のモノクローナル抗体を作出し、その性状の解析を行った（第４章)。最後に CDV ゲノムの性状を電子顕微鏡下での解析から新たな知見が得られたのでその報告も行った（第５章)。

第一章：EGFP 発現ＣＤＶを用いたイヌ海馬における CDV 感染動態の解析

イヌの CDV 感染症では中枢神経系への感染が多く認められるが、中枢神経系における CDV の感染拡大様式は明らかにされていなかった。本章では、感染細胞で蛍光を発する EGFP-CDV をイヌ海馬スライスに感染させ、中枢神経系での CDV の感染動態を解析した。ビブラトームでスライス状にした海馬スライスを器官培養して３週間後にウイルス液を滴下し自然感染させた。感染動態は共焦点顕微鏡下で蛍光を指標として経時的に観察した。CDV 感染後、２４時間目には既に蛍光が観察され経時的に周囲の細胞へと伝播した。ニューロン、アストロサイト、オリゴデンドロサイトに対する特異的マーカー抗体でそれぞれ免疫染色を行ったところ、感染細胞の 80%以上がニューロンであることが明らかになった。ニューロンでの感染動態を経時的に観察したところ、シナプスを介して隣接するニューロンに感染していることがわかった。ニューロンでのウイルス膜蛋白の動態を特異抗体を用いた免疫染色で調べたところ、F と H はニューロン全体に分布しているのに対して、M は細胞体の他に一部の軸索やシナプスでのみ発現がみられた。感染３ヶ月目に海馬スライスを電子顕微鏡下で観察したところ、シナプス末端でリボヌクレオキャプシドの蓄積がみられ、更にシナプス外にウイルス粒子を観察することができた。これらの結果から、CDV は中枢神経系ではシナプス末端からシナプス外にウイルス粒子を放出し、隣接するニューロンのシナプスを介してウイルスの侵入・伝播が起こると考えられた。

第二章：デリバリーベクターとしての CDV の利用：抗酸化治療に向けたイヌ SOD1 発現組換え CDV の作出

酸化ストレスは神経障害の主な原因と考えられ、標的細胞への抗酸化酵素 SOD の導入は効果的な治療法の一つとして期待されている。本章では CDV リバースジェネティクス系によりイヌ SOD1 発現組換えウイルスの作出を行った。イヌ SOD1 遺伝子をクローニングし、CDV リバースジェネティクス系に従ってウイルスレスキューを行い、SOD1-CDV の作出に成功した。感染細胞での CPE の形状やウイルス増殖曲線は元株 Yanaka 株と比較し顕著な相違は認められなかった。western blot と SOD 活性測定により感染細胞で SOD 活性を持つ機能的な蛋白が十分量発現していることがわかった。第１章では、CDV はニューロンに対して高い感受性があること

が明らかになった。本章での結果から、SOD1-CDV はニューロンに感染し機能的な SOD1 を産生するデリバリーベクターとしての有用性が示唆された。

第三章：CDV およびリーシュマニア感染症に対する組換え CDV 二価ワクチンの開発

リーシュマニア原虫症はサシチョウバエを媒介昆虫とし、ヒトやイヌ等に感染して皮膚および内臓に重篤な病巣を形成する人獣共通感染症である。ヒトでは熱帯の発展途上国を中心に毎年 4 千万人以上の感染例が報告されている。この保有宿主ともなるイヌで感染を防ぐことはヒトリーシュマニア症の対策としても有用と考えるが、未だ有効なワクチンは開発されていない。本章では、原虫に対する有効な多価ワクチンの開発を目的として、リーシュマニア抗原を発現する組換え CDV の作出を試みた。リーシュマニア抗原として TSA, LmSTI1 を選択し、CDV リバースジェネティクス系により組換え CDV (TSA-CDV, LmSTI1-CDV)の作出に成功した。これまでに作出成功した LACK-CDV と同様、この２つの組換え CDV は元株 Yanaka 株と比べ、ウイルス増殖速度・CPE の形状に顕著な差異は認められなかった。特異抗体を用いた解析により、組換え CDV 感染細胞において抗原蛋白の発現が確認できた。これら組換え CDV を６週齢の幼犬に静脈内接種したところ、体温・リンパ球数・臨床所見においても変化がなく、組換え CDV は病原性がなく安全であることが証明できた。CDV ワクチンとしての有効性は既に LACK-CDV 接種犬が CDV 強毒株チャレンジに対して完全な抵抗性を示したことから証明している。リーシュマニアチャレンジ実験では、リーシュマニア接種後 10 週目まで、接種部位で形成される結節の大きさを指標としてリーシュマニア増殖抑制能を検討した。抵抗性誘導能は LACK-CDV で最も顕著であったが、TSA-CDV, LmSTI1-CDV, LACK-CDV を混合して接種したイヌでは結節形成の増大を抑制するのみでなく消失も早め、有効性を示した。これらの結果から、本章で作出した組換え CDV はリーシュマニア症に対する多価ワクチンとしての有効であると考えられた。

第四章：CDV P 蛋白に対するモノクローナル抗体による認識部位の検索

モービリウイルスゲノムは 6 つの構成遺伝子をコードし、その一つである P 遺伝子からは P、V、C の蛋白が翻訳される。P 蛋白はこれまでウイルスの転写や複製に必要であることが知られているが最近病原性の発現に関わっていることが報告されている。本章では、P 蛋白の機能解析ツールとして CDV Yanaka 株 P 蛋白に対するモノクローナル抗体を作出しその解析を行った。P とV蛋白は一部同一のアミノ酸配列を持つため、P 特異的な領域について組換え蛋白を作出し、常法に従ってハイブリドーマを作出して、最終的に 7 つのモノクローナル抗体を得た。これら抗体間の交差反応性を調べたところ、２つのグループ（I: 13Ea,52G,36Ba,42Ba,99Bb, II: 33Ba,36E）に分かれた。更にモービリウイルス間での交差反応性を調べたところ、I 型は Yanaka 株と系統的に近縁なハクビシン由来 CDV Haku93 株と Haku00 株と、更に 99Bb 以外の I 型は RPV (RBOK 株, L 株)とも反応し、I 型は更に２つに分けられることがわかった。一方、得られた抗体は全て CDV Snyder Hill 株, Onderstepoort 株及び MV Edmonston 株, HL 株に対して反応を示さなかった。以上の結果から、P 蛋白特異的領域には少なくとも三つの抗体認識部位が存

在することがわかった。

第五章：CDV 粒子内ゲノムの電子顕微鏡学的解析

モービリウイルスでは、ウイルスゲノムの動態や形態学的な性状は感染細胞内に比べて、ウイルス粒子内についてはよくわかっていない。本章ではウイルス粒子内にパッケージングされた CDV ゲノムの形態学的性状について解析を行った。感染細胞から放出されるウイルス粒子を濃縮した培養液からショ糖密度勾配を用いて超遠心分離で分画し、ウイルス分画を電子顕微鏡下で解析した。ウイルス粒子に取り込まれるウイルスゲノム全長はこれまで報告されていた 1000nm よりも極めて長いものが多く観察され、一つのウイルス粒子内に複数のウイルスゲノムが取り込まれている可能性が示唆された。更にフィラメント状のウイルスゲノムは主に直径が太いタイプ（long filamentous type: LF）で、一部で、細いタイプ（small filamentous type: SF）が存在することがわかった。第４章で作出した CDV P 蛋白に対するモノクローナル抗体を用いて免疫電子顕微鏡法で解析したところ、P 蛋白は SF の表面には存在せず、一方 LF 上では全体を覆っていることがわかった。これらの結果から、ウイルス粒子内にパッケージングされたウイルスゲノムは多様な形態を構成していることが明らかになった。

本研究では、CDV リバースジェネティクス系によって作出した組換え EGFP-CDV による中枢神経系での CDV 感染動態の検索、デリバリーウイルスとして利用可能なイヌ SOD1 発現する組換え CDV の作出、更に有効な二価ワクチンウイルスとしてリーシュマニア抗原を発現する組換え CDV の有用性を示すことができた。一方将来的にはより安全で有効なウイルスベクターの開発のためにも CDV の基礎的な研究を行い、病原性やウイルス複製に関与する CDV P 蛋白に対するモノクローナル抗体を作出し、電子顕微鏡解析によりウイルス粒子内のゲノムの形態で新しい知見を得ることができた。本研究成果は、CDV の基礎的な新知見のみならずウイルスベクター開発を大きく進展させる有用な知見や成果を与えるものであると考える。