Treatment of rabies by intrathecal immunization and pathogenesis of myocardial necrosis in rabid rabbits

(鞘内免疫による狂犬病の治療と狂犬病発症ウサギに見られた心筋症の病理発生)

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Contents

List of abbreviations

ANOVA	analysis of variance
BBB	blood brain barrier
BHK	baby hamster kidney
BSA	bovine serum albumin
BSAT	bovine serum albumin with Tween-20
BSL2	biosafety level 2
°C	Celsius or centigrade
CD	cluster of differentiation
CGRP	calcitonin-gene related peptide
CNS	central nervous systems
CO_2	carbon dioxide
CSF	cerebrospinal fluid
CVS	challenge virus standard
CXCL12	chemokine (C-X-C) ligand 12
DAB	3,3'- diaminobenzidine
DNA	deooxyribonucleic acid
dpi	days post-inoculation
ELISAs	enzyme-linked immunosorbent assays
FFU	focus forming unit
FITC	fluorescein isothiocyanate
G	glycoprotein
GFAP	glial fibrillary acidic protein
HE	hematoxylin and eosin
hr	hour

HPRT	hypoxanthine phosphoribosyl transferase
H_2SO_4	sulfuric acid
Iba-1	ionized calcium-binding adaptor molecule-1
ID	identification
IFN	interferon
IgG	immunoglobulin G
IHC	immunohistochemistry
IT	intrathecal
IU	international unit
KCl	potassium chloride
kg	kilogram
L	large subunit of the RNA-dependent RNA polymerase
M	matrix protein
M	molar
mg	milligram
ml	milliliter
n	number
N	nucleoprotein
nm	nanometer
Nos	numbers
OD	optical density
P	phosphoprotein
PBS	phospate buffer saline
PBST	phosphate buffer saline with 0.05% Tween-20
PEP	post-exposure prophylaxis

RABV	rabies virus
RFFIT	rapid fluorescent focus inhibition test
RIGs	rabies immunoglobulins
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SAB-PO	streptavidin-biotin-peroxidase system
SC	subcutaneous
SD	standard deviation
STAT	signal transducer and activator of transcription
TC	tissue culture
TMB	3,3',5,5'-tetramethylbenzidine
Tris-HCl	tris(hydroxymethy)aminomethane-hydrochloride
Tx	treatment
W	watt
WHO	World Health Organization
VNA	viral neutralizing antibody
µg	microgram
µl	microliter

General introduction

Rabies is progressive encephalomyelitis that is one of the oldest zoonotic diseases and causes approximately 55,000 fatalities annually (WHO, 2005). The disease is invariably fatal when rabies virus (RABV) invades the brain (Rupprecht et al., 2010) in almost mammals (Dietzschold et al., 2008).

RABV belongs to genus *Lyssavirus* in the family *Rhabdoviridae* (Ivanov et al., 2011) and is classified into 2 strains, fixed and street strains, based on their origin (Tsiang and Guillon, 1981) and they have different pathogenicities. RABV is non-segmented, single strand, negative sense RNA virus. The viral genomes encode at least 5 viral proteins including nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large subunit of the RNA-dependent RNA polymerase (L), and they are oriented 3′-N-P-M-G-L-5′ (Ivanov et al., 2011) in order. The helical viral RNA is associated with P and L proteins and enwrapped by N protein, to form ribonucleoprotein (RNP) which functions as a template for viral transcription and replication (Shimizu et al., 2007). While P and L proteins act as RNA-dependent RNA polymerase (Shimizu et al., 2007).

The precise pathogenesis of rabies is still mysterious. However, neurotropism, neuroinvasion and immunoevasion are documented as the pathological mechanisms of rabies, that is, viral entry, transaxonal transportation, trans-synaptic cell-to-cell transportation and the ability of viral replication play the crucial role for neuroinvasion in RABV infection (Dietzschold et al., 2008). Transdermal inoculation is the main infection route of RABV (Dietzschold et al., 2008), which replicates firstly in the myocytes (Lewis et al., 2000) at the infected site before invading into the spinal cord via sensory and motor nerves (Lewis et al., 2000; Park et al., 2006). The G protein is the viral protein that is expressed on the cell surface and used to interact with highly specific neuronal receptor including nicotinic acetylcholine receptor, neuron adhesion molecule and p75 neurotrophin receptor (Klingen et al., 2008). The

virus becomes incorporated by endocytosis pathway into the cell (Lewis et al., 2000) and complete their transcription and replication in the specific replication machinery called Negri body that located in the neuronal cytoplasm, which is a histological hallmark for RABV infection (Albertini et al., 2008). The G protein also forms the complex with RNP and M protein, particularly the PPEY motif (Wirblich et al., 2008), to control the viral budding (Shimizu et al., 2007). The expression level and conformation of the G protein are the critical factor for G protein-associated pathogenicity and immunoevasion (Wirblich and Schnell, 2011). The magnitude of G protein expression is different upon the strains of RABV (Miyamoto and Matsumoto, 1967; Tsiang and Guillon, 1981; Zhang et al., 2013) and remarkable expression of G protein in fixed strain causes immune-mediated mechanism (Zhang et al., 2013). The G protein interacts with M proteins to contribute for trans-axonal transportation of completely enveloped RABV particle (Kleingen et al., 2008; Pulmanasuhakul et al., 2008). The expression level of G protein is tightly controlled and optimally interacts with another protein and viral genome to maintain the pathogenicity (Morimoto et al., 2000; Pulmanasuhakul et al., 2008). The T lymphocyte-dependent injury is also considered as a mechanism in rabies paralysis (Johnson et al., 2008; Sugiura et al., 2011). Axonal injury is caused by oxidation in RABV infection (Jackson et al., 2010). In addition, neuronal dysfunction, overexpression of chemokine, and neuropeptide, in particularly calcitonin-gene related peptide (CGRP), are also proposed as crucial factors for rabies pathogenicity (Dhingra et al., 2007; Fu and Jackson, 2005; Yan et al., 2001).

Although, immune response plays the pivotal role to eliminate RABV from host (Chopy et al., 2011; Galelli et al., 2000; Hooper 2005), recent virological studies have revealed that street RABV has anti-apoptotic and anti-inflammatory effects that suppress nerve cell damage and inflammatory responses in the central nervous systems (CNS) of animals heavily infected with RABV (Dhingra et al., 2007; Fernandes et al., 2011; Morimoto et al., 1999; Morimoto et

al., 2000; Moseley et al., 2009; Suja et al., 2009; Yan et al., 2001: Xiaohu et al., 2011). P protein of RABV interferes the interferon (IFN) production by inhibiting nuclear translocation of signal transducer and activator of transcription 1 (STAT1) and STAT2 (Ito et al., 2010; Moseley et al., 2009; Vidy et al., 2007). RABV N protein submerges the retinoic acid-inducible gene 1-mediated innate immune response (Masatani et al., 2010). RABV can infect neuron without triggering apoptosis of the infected neuron by limited expression level of G protein (Faber et al., 2004). On the other hand, RABV induces apoptosis of the infiltrative adaptive immune cells (Fernandes et al., 2011; Lafon et al., 2008).

Although post-exposure vaccination accompanied with local administration of rabies immunoglobulins (RIGs) and wound cleaning is effective at preventing rabies after exposure to the virus (Warrell, 2012), the timing of the therapy is crucial to success (Warrell, 2012; Weideman et al., 2012; Willoughby et al., 2005). No effectively therapy is available for rabies so far (Jackson et al., 2006). Two rabid human patients fortunately survived after being put into a therapeutic coma using anesthetic agents and antiviral drugs, and the treatment is called "Milwaukee protocol" (Weideman et al., 2012; Willoughby et al., 2005). However, the protocol was not successful in other patients, and the scientific rationale of the Milwaukee protocol remains controversial (Hemachudha et al., 2006; Hunter et al., 2010; Jackson et al., 2006).

Previously, it had been reported that intrathecal (IT) immunization, which involves the direct inoculation of antigens into the cerebrospinal fluid (CSF), induced specific antibodies against RABV in the CSF (Aoshima et al., 2011; Shin et al., 2009). The immunization was effective at inducing a protective immune response against the transneural spread of RABV (Aoshima et al., 2011; Shin et al., 2009) and suppressed the spread of intracerebrally inoculated RABV in mice (Sunden et al., 2010). In addition, subcutaneous (SC) immunization prior to IT immunization (SC/IT immunization) induced a more rapid and higher antibody

response in the CSF than IT immunization alone. The antibody detected in the CSF was composed of antibodies derived from the serum as well as *de novo* antibody produced locally in the brain (Aoshima et al., 2010). In Chapter 1, I describes for the first time that repeated IT immunization of rabbits showing clinical symptoms of rabies can clear RABV from the CNS of the animals and prevent fatality. I believe the therapeutic protocol described here may provide a foundation for the future development of novel therapies clinically applicable to rabies.

Cardiac complication has been described in rabies patients (Cheetam et al., 1970; Feiden, 2013; Jackson, 2011; Metze and Feiden, 1991) and the pathogenesis of the lesion has been attributed to direct viral replication or inflammation of the nerve in the heart (Feiden, 2013). RABV spreads centrifugally to the heart via innervation or viremia (Cheetam et al., 1970; Feiden, 2013). However, cardiac complication has not been reported in the rabid animals. In my treatment experiments for rabies, I found frequent occurrence of myocardial necrosis in rabid rabbits. In Chapter 2, I describes the pathogenesis of the myocardial necrosis.

Chapter 1

Survival of rabid rabbits after intrathecal immunization

Introduction

Rabies is one of the oldest zoonotic diseases and causes approximately 55,000 fatalities annually. Rabies is invariably fatal when rabies virus (RABV) invades the brain (Rupprecht et al., 2010). High levels of viral neutralizing antibody (VNA) have been found in the cerebrospinal fluid (CSF) in a few of cases recovered from rabies (Hamir et al., 2011; Jackson, 2006; Tillotson et al., 1977; Wiedeman et al., 2012; Willoughby et al., 2005). Two rabid human patients survived after being put into a therapeutic coma under the so-called "Milwaukee protocol" (Wiedeman et al., 2012; Willoughby et al., 2005). However, therapeutic coma was not successful in other patients, and the scientific rationale of the Milwaukee protocol remains controversial (Hemachudha et al., 2006; Hunter et al, 2010; Jackson, 2006).

Recent virological studies have revealed that RABV has anti-apoptotic and anti-inflammatory effects that suppress nerve cell damage and inflammatory responses in the central nervous systems (CNS) of heavily infected animals (Fernandes et al., 2011; Suja et al., 2010; Wang et al., 2011; Yan et al., 2001). These features imply that the elimination of RABV from the CNS by appropriate treatment could lead to recovery from rabies.

It has been reported that intrathecal (IT) immunization, which involves the direct inoculation of antigens into the CSF, induced specific antibodies against RABV in the CSF (Aoshima et al., 2011; Shin et al., 2009). The immunization induced a protective immune response against the transneural spread of RABV (Aoshima et al., 2011; Shin et al., 2009) and suppressed the spread of intracerebrally inoculated RABV in mice (Sunden et al., 2010). Subcutaneous (SC) immunization prior to IT immunization induced a more rapid and higher

antibody response in the CSF than IT immunization alone (Aoshima et al., 2011). In this chapter, I report here for the first time that repeated IT immunization of rabbits showing clinical symptoms of rabies can clear RABV from the brain and prevent fatality. The protocol may rescue the life of rabid patients and prompt the future development of novel therapies against rabies.

Materials and Methods

Virus

The challenge virus standard (CVS) strain, a neurovirulent, fixed RABV (Sugiura et al., 2011) strain, was used in the present study since this virus does not descend from the CNS to the periphery and is safe to handle in a laboratory setting, unlike street RABV (Miyamoto and Matsumoto, 1967; Sugiura et al., 2011). Furthermore and more importantly, the latent period between virus inoculation and the clinical appearance of rabies is fixed at about one week for the CVS strain.

Animals and experimental design

A total of 27 conventional clean New Zealand White rabbits (16-week-old, Japan SLC Inc., Japan) were used in this study. Twenty-four rabbits were injected subcutaneously with 1 ml of the inactivated whole RABV particles (Nisseiken Rabies tissue culture (TC) vaccine, Nisseiken Co., Japan) into the dorsal subcutis. Three days later, the rabbits were intramuscularly inoculated with RABV in both hind limbs with 2 ml of inoculums (4×10^7 focus forming unit (FFU)/ml). Ten of the 24 rabbits (41.7%) showed neuromuscular symptoms of rabies prior to 8 days post inoculation (dpi) and remaining 14 rabbits were excluded from the experiment. These ten rabbits were allocated into three groups. Three rabbits received no further treatment after showing symptoms of rabies (the SC group); three rabbits received 3 additional SC immunizations (the SC/SC group) using the vaccine and four

rabbits were treated with 3 additional IT immunizations (SC/IT immunization) on days 1, 2, and 4 after showing symptoms of rabies. For IT immunization, the rabbit was inoculated with 1 ml of the vaccine into the subarachnoid space via *cisterna cerebellomedullaris* immediately after collecting 1 ml of CSF under anesthesia using xylazine hydrochloride (2 mg/kg Selactar; Bayer Health Care, Germany) and ketamine hydrochloride (35 mg/kg Ketalar; Daiichi Sankyo Co., Japan). An additional three naïve rabbits were inoculated intramuscularly with RABV and no vaccination was given (the non-treatment group; see Figure 1.1 for the treatment schema). All the recumbent rabbits were given daily injections of 100-150 ml of saline containing 5% glucose and 10 ml of amino acid solution (Aminoleban, Otsuka Pharmaceutical Co., Japan) through the ear vein. Surviving rabbits were kept up to 28 days after showing rabies symptoms and were euthanized by exsanguination under deep anesthesia using xylazine hydrochloride and ketamine hydrochloride.

Antibody measurements

Serum and CSF were collected at each time point shown in Figures 1.4 and 1.5 and were stored at -20°C until antibody titers were assayed. The serum and CSF were heat inactivated at 56°C for 30 min to inactivate the complement before apply for VNA assay and ELISAs. The VNA assay was performed using a rapid fluorescent focus inhibition test (RFFIT), as previously described (Hamir et al., 2011; Wang et al., 2011). Serum and CSF were diluted with maintenance medium at two fold and made a serial dilution in 96-well tissue culture plate. The plates were added with 50 µl of viral suspension (CVS-11 strain of RABV, 100 tissue culture infective $dose_{50}$) and incubated at 37°C for 90 min in CO_2 incubator. The serial dilution of WHO reference anti-RABV antibody standard was used as a positive control and PBS was used as a negative control. Both positive and negative control was analyzed in accompanied with the test samples. The mixture of virus and serum or CSF was transferred to 96-well tissue culture plate, which seeded with baby hamster kidney (BHK) cells, and

incubated at 37°C overnight in CO_2 incubator. Then, the plates were fixed with 90% acetone. To detect RABV-infected cells, the plates were stained with FITC-labeled anti-RABV conjugate at 37°C for 30 min. VNA titer was determined as an inverse of the highest dilution of serum or CSF that neutralized 50% of the RABV and normalized to international unit (IU)/ml with WHO reference anti-RABV antibody standard. Enzyme-linked immunosorbent assays (ELISAs) were conducted as previously described (Aoshima et al., 2011). Each well of ELISA plates (BD Falcon, Franklin Lakes, NJ, USA) were coated with 50 µl of rabies antigen [inactivated whole RABV particles (Nisseiken Rabies TC Vaccine, Nisseiken Co., Japan)] treated with disruption buffer containing 0.05 M Tris-HCl (pH 7.8), 0.5% Triton X-100, and 0.6 M KCl for 2 hr at room temperature (RT). The excessive antigen was discharged. The plates were washed three times with PBST (phosphate buffered saline with 0.05% Tween-20) and blocked with 100 µl of 1% BSA fraction V (Sigma-Aldrich Japan, Tokyo, Japan) in PBS for 1 hr at RT. The inactivated serum and CSF were diluted in 0.5% BSAT (0.5% BSA fraction V in PBST), added to each well for 50 µl in duplicate, and incubated for 1 hr at RT. 0.5% BSAT was used as a negative control. The washed plates were added with 50 µl in each well of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:5000; Invitrogen, Carlsbad, CA, USA) diluted in 0.5% BSAT and incubated for 1 hr at RT. The solution was discharged and plates were then washed three times. TMB substrate (TMB substrate Kit, Thermo Fisher Scientific, MA, U.S.A.) was added to each well at 100 µl for 20 min and the reaction was stopped by adding 100 µl of 2 M H_2SO_4. The optical density (OD) was read at 450 nm in a Thermo scientific multiscan (Thermo Fisher Scientific)

Histopathology and immunohistochemistry

Selected tissues, including visceral organs and nervous tissues, were collected and fixed in 20% buffered formalin. The fixed tissues were embedded in paraffin wax and cut into 4 µm thickness. The sections were stained with hematoxylin and eosin for histopathological

examination. For immunohistochemistry (IHC), a streptavidin-biotin-peroxidase system (SAB-PO Kit; Nichirei Bioscience, Japan) was employed. Primary antibodies used for IHC were monoclonal mouse anti-rabies neucleoprotein (clone N13-27; kindly provided by Dr. Naoto Ito, Gifu University), monoclonal mouse anti-human glial fibrillary acidic protein (GFAP) (clone 6F2; DAKO, USA), monoclonal mouse anti-human CD3 (clone F7.2.38; DAKO), monoclonal mouse anti-human CD79α (clone MH57; DAKO), and goat polyclonal anti-rabbit Iba-1 (code ab5076; Abcam, UK). Briefly, the deparrafiniztion and rehydration were performed using xylene and graded alcohol, respectively. Antigen retrieval using heat treatment was employed in buffer citrate at 95°C for 15 min with 750 W microwaves. The endogenous peroxidase was inactivated by 0.3% H_2O_2 in methanol for 15 min at RT. The sections were applied with 10% normal rabbit serum and 10% normal mouse brain homogenate (for RABV antigen detection) to block the nonspecific reaction for 15 min at RT. The primary antibodies were diluted with PBS at 1:1,000 for anti-rabies nucleoprotein, 1:100 for GFAP, 1:50 for CD3, 1:50 for CD79α, and 1:100 for Iba-1 and incubated at 4°C overnight. The negative control section was incubated with PBS instead of primary antibody. After washing, the sections were applied with secondary antibody (anti-mouse IgG antibody, Nichirei Bioscience). Then, avidin-complex was implement for 5 min at RT. The positive signal was visualized with 3,3'-diaminobenzidine (DAB, DAKO). Mayer's haematoxylin was used for counterstaining.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from brain tissue using the RNeasy Kit (Qiagen, USA) and 5 μg of RNA was used for reverse transcription with the Superscript First-Strand Synthesis system (Life Technologies, USA). The fragment of the RABV genome encoding matrix protein was amplified using Go Taq DNA polymerase (Promega, USA) and the following primer pairs: F, 5'-GTC GAC ATG AAC GTT CTA CGC AAG ATA G-3'; and R, 5'-GCG GCC GCT TAT

TCT AGA AGC AGA GAA G-3'. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control.

Statistical analysis

Statistically significant differences in antibody levels between surviving and non-surviving rabbits were evaluated by repeated measures analysis of variance (ANOVA) and significance was set at $p < 0.05$.

Ethical statement

All animal experiments were conducted in the BSL2 facility of Hokkaido University Research Center for Zoonosis Control after approval of the Animal Care and Use Committee of the Hokkaido University (Approval number 09-0028).

Results

Clinical findings

Ten of the 24 rabbits (41.7%) showed neuromuscular symptoms of rabies prior to 8 dpi and remaining 14 rabbits were excluded from the experiment. The clinical course of the development of rabies symptoms and rabies lethality is summarized in Table 1 and Figure 1.2. All the 10 rabbits showed clinical signs of rabies, including an unstable gait, lack of coordination of the hind limbs, gradual decreases in food intake and water consumption, and increased salivation and lacrimation, within 4-8 dpi. After this period, the rabbits progressively developed tetraplegia, lateral recumbency, and generalized spasms, at 8-10 dpi. All three rabbits in the non-treatment group, two of three rabbits in the SC group, and all three rabbits in the SC/SC group died within 8-12 dpi. On the other hand, one rabbit in the SC group and all four rabbits in the SC/IT group recovered from the terminal stage and resumed drinking and eating. They began responding to external stimuli again at 12-18 dpi and survived until the end of the study (Figure 1.2). However, they remained recumbent, with

decreased body weight (Figure 1.3), and did not regain the ability to stand up or walk during the observation period.

Antibody response

VNA and ELISA antibody titers in the serum and CSF increased gradually after RABV inoculation and peaked at the end of the experiment in surviving rabbits (Figures 1.4 and 1.5). At the time of the third treatment (7-10 dpi), antibody titers were not significantly different between the rabbits that ultimately survived and those that did not.

Pathological findings and viral genome detection

Macroscopically, the surviving rabbits showed muscular atrophy and a decreased amount of subcutaneous and abdominal adipose tissues.

Microscopically, the eight rabbits that died (three from the non-treatment group, two from the SC group, and three from the SC/SC group) had neuronal necrosis with occasional neuronophagia and deposition of a large amount of RABV antigen in the nerve cell bodies/projections throughout the CNS. These changes were most prominent in the cerebral cortex of the parietal lobe, the thalamus, the hypothalamus, the nuclei of ascending pathway and reticular formation of the brain stem, the vermis of the cerebellum (Figure 1.6a), the dorsal horn and intermediate substance of gray matter of the spinal cord, and the lumbar and sacral dorsal ganglia. Proliferation and hypertrophy of Iba-1^+ microglia and diffuse perivascular and meningeal lymphocytic infiltrates consisting mainly of $CD3^+$ T lymphocytes were also observed in these tissues. A small number of $CD79^+$ B lymphocytes infiltrated in the meniges, perivascular spaces of the CNS and dorsal ganglia, and T lymphocytes were dominant over B lymphocytes in number at any of the areas. In contrast, descending motor and pyramidal nerve routes such as the cerebral basal nuclei, the hippocampus, the cerebellar hemisphere, and the ventral horn of the spinal cord were relatively spared from severe pathological changes.

In the five rabbits (one in the SC group and four in the SC/IT group) that survived the virus challenge, nerve cell loss was prominent for Purkinje and granular cells of the cerebellar vermis (Figure 1.6b), the pontine reticular nuclei and tegmental areas of the brain stem, and the dorsal horn and intermediate substance of the spinal cord. Small malacic foci were also sometimes observed in these areas. These changes were accompanied by mild hyperplasia of Iba-1^+ microglia, $GFAP^+$ astrocyte swelling, and low levels of lymphoplasmacytic infiltration in the meninges, perivascular space, and nerve tissues. The lymphoplasmacytic infiltrate was composed mainly of T lymphocytes and B lymphocytes were presented in small numbers. RABV antigen was rarely detected either in the CNS (Figure 1.6b) or in the peripheral nervous tissues of surviving rabbits. The RABV matrix gene was detected by RT-PCR in the brain tissue of non-surviving rabbits but was not found in the brains of surviving rabbits (Figure 1.7).

Discussion

The significance of high VNA titers in the CSF has been emphasized in humans and animals after recovery from rabies (Hamir et al., 2011; Miller et al., 1978; Willoughby et al., 2005), and VNA is considered as a crucial factor for recovery (Hooper et al., 1998, 2009; Liao et al., 2012; Miller et al., 1978; Perry and Lodmell, 1991). It has been reported that IT immunization induced specific antibodies against RABV in the CSF and a protective immune response against the transneural spread of RABV (Aoshima et al., 2011; Shin et al., 2009). The immunization could suppress the spread of intracerebrally inoculated RABV in mice (Sunden et al., 2010). SC immunization prior to IT immunization induced a more rapid and higher antibody response in the CSF than IT immunization alone (Aoshima et al., 2011). Based on these previous findings, I tried a treatment of rabid rabbit using SC/IT immunizations. In the present study, VNA and ELISA antibody titers in the serum and CSF

were markedly elevated in surviving rabbits at the end of the study. However, the antibody responses of rabbits that ultimately survived and those that died were not significantly different at the peak of clinical symptoms (8-12 dpi) (i.e., the time at which the non-surviving rabbits died), and the lymphocytic infiltrate in the CNS of rabbits that did not survive consisted predominantly of T lymphocytes. These findings indicate that the antibody titers in the serum and CSF are not the sole factors mediating the clearance of RABV from the CNS.

RABV antigen directly injected into the CSF of the brain drains into the deep cervical lymph nodes and stimulates the production of RABV-specific antibodies and cytotoxic T lymphocytes in the spleen (Stevenson et al., 1997). IT immunization also increases the permeability of the blood-brain barrier (BBB) and allows for the migration of effector cells into the CNS (Phares et al., 2006; Wang et al., 2011). The B lymphocytes infiltrating the CNS via up-regulation of the chemokine CXCL12 (Lee et al., 2012) are the source of locally produced antibody important in the clearance RABV from the CNS (Aoshima et al., 2011; Hamir et al., 2011; Hooper et al., 2009; Lee et al., 2011). In addition, effector T lymphocytes infiltrating the CNS permit the clearance of RABV by inducing apoptosis of infected neurons in the presence of antibody (Galleli et al., 2000). These previous reports suggest that a combination of both humoral and cellular immunities (Dietzschold et al., 1992; Hooper et al., 1998; Phares et al., 2006; Wang et al., 2011) contributed to the clearance of RABV from the CNS in the present study.

The present results clearly showed that the rabid rabbits treated by repeated IT immunization after SC immunization could tolerate the peak of the rabies symptoms and recover incompletely thereafter. For human patients ever received pre- or post-exposure vaccination, it might be a possible therapeutic strategy to encourage the effective immune response in the CNS by IT immunization combined with intensive care or coma (Willoughby et al., 2005) to secure efficient time. However, the outcome of the treatment of the present

study was limited because (1) the damage to the CNS tissue of surviving rabbits was too severe to allow complete recovery, and (2) a neurovirulent, fixed RABV (CVS strain) was used instead of street RABV. Most humans who survived rabies showed severe neural complications after recovery (Tilloson et al., 1977) and prompt initiation of IT immunizations may benefit recovery from rabies with mild neurological complications. The CVS strain causes G protein to be expressed on the surface of infected neurons (Faber et al., 2002; Miyamoto and Matsumoto, 1967), which induces apoptosis of the infected neurons and neighboring cells, as well as inflammation in the infected brain (Faber et al., 2002; Galleli et al., 2000; Miyamoto and Matsumoto, 1967; Sugiura et al., 2011). On the other hand, street RABV induces low levels of G protein on the surface of infected neurons due to post-transcriptional modification (Yan et al., 2001). The anti-apoptotic, anti-inflammatory and immunosuppressive effects of street RABV infection allow the virus to infect neurons without cellular destruction or inflammation (Fernandes et al., 2011; Suja et al., 2011). These findings indicate that the elimination of street RABV from the CNS by IT immunization may lead to a more satisfactory recovery from RABV-induced rabies than from that caused by the CVS strain.

Summary

Rabies is a fatal zoonotic disease for which no effective treatment measures are currently available. Rabies virus (RABV) has anti-apoptotic and anti-inflammatory properties that suppress nerve cell damage and inflammation in the central nervous systems (CNS). These features imply that the elimination of RABV from the CNS by appropriate treatment could lead to complete recovery from rabies. Ten rabbits showing neuromuscular symptoms of rabies after subcutaneous (SC) immunization using commercially available vaccine containing inactivated whole RABV particles and subsequent fixed RABV (CVS strain) inoculation into hind limb muscles were allocated into three groups. Three rabbits received no further treatment (the SC group), three rabbits received three additional SC immunizations using the same vaccine, and four rabbits received three intrathecal (IT) immunizations, in which the vaccine was inoculated directly into the cerebrospinal fluid (CSF) (the SC/IT group). An additional three naïve rabbits were inoculated intramuscularly with RABV and not vaccinated. The rabbits exhibited neuromuscular symptoms of rabies within 4-8 days post-inoculation (dpi) of RABV. All of the rabbits died within 8-12 dpi with the exception of one rabbit in the SC group and all four rabbits in SC/IT group, which recovered and started to respond to external stimuli at 11-18 dpi and survived until the end of the experimental period. RABV was eliminated from the CNS of the surviving rabbits. Antibody response in serum and CSF was not significant different between survived rabbits and dead rabbits at 8-12 dpi. The results suggest that humoral and cellular immunities in CNS were involved in RABV clearance and recovery. Although my treatment protocol was still incomplete as an accomplished therapy for rabies, the present results indicate that IT immunization can at least rescue the life of rabid patients and prompt the future development of novel therapies against rabies.

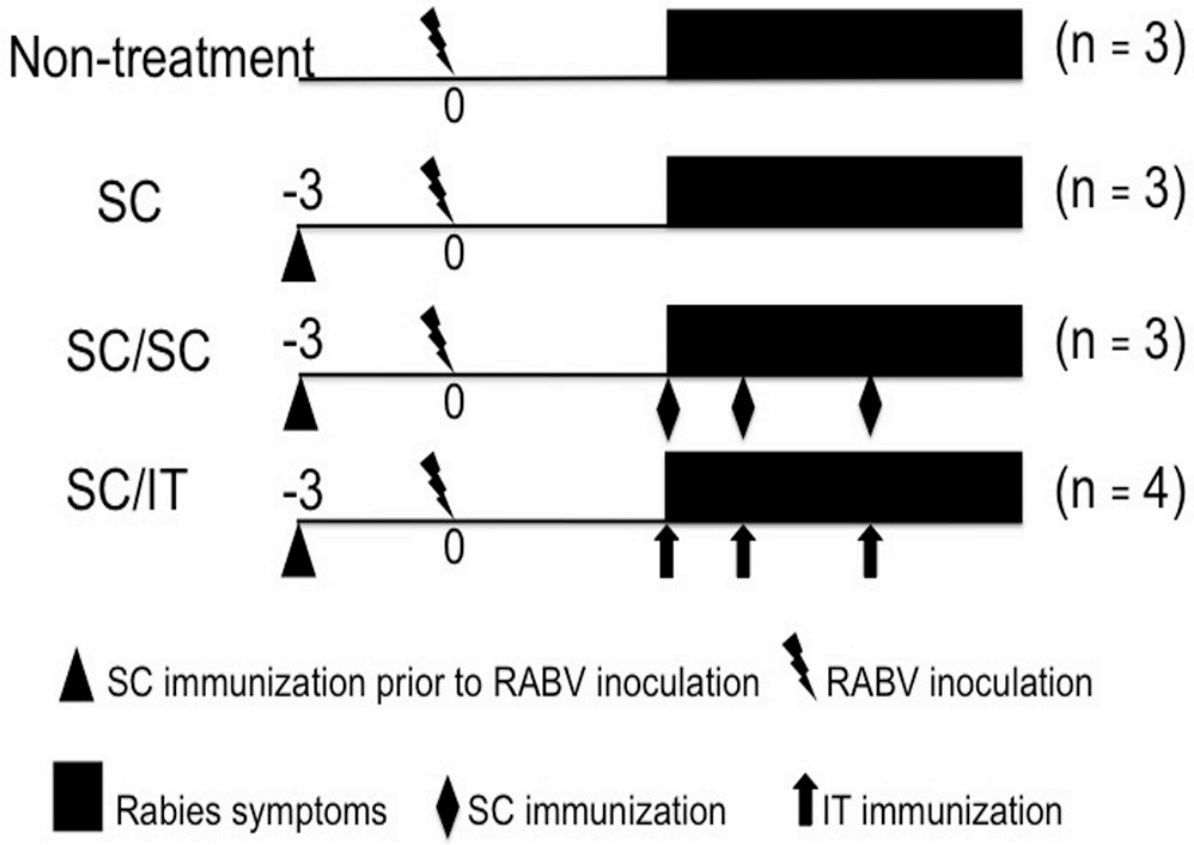

Figure 1.1. Experimental protocol.

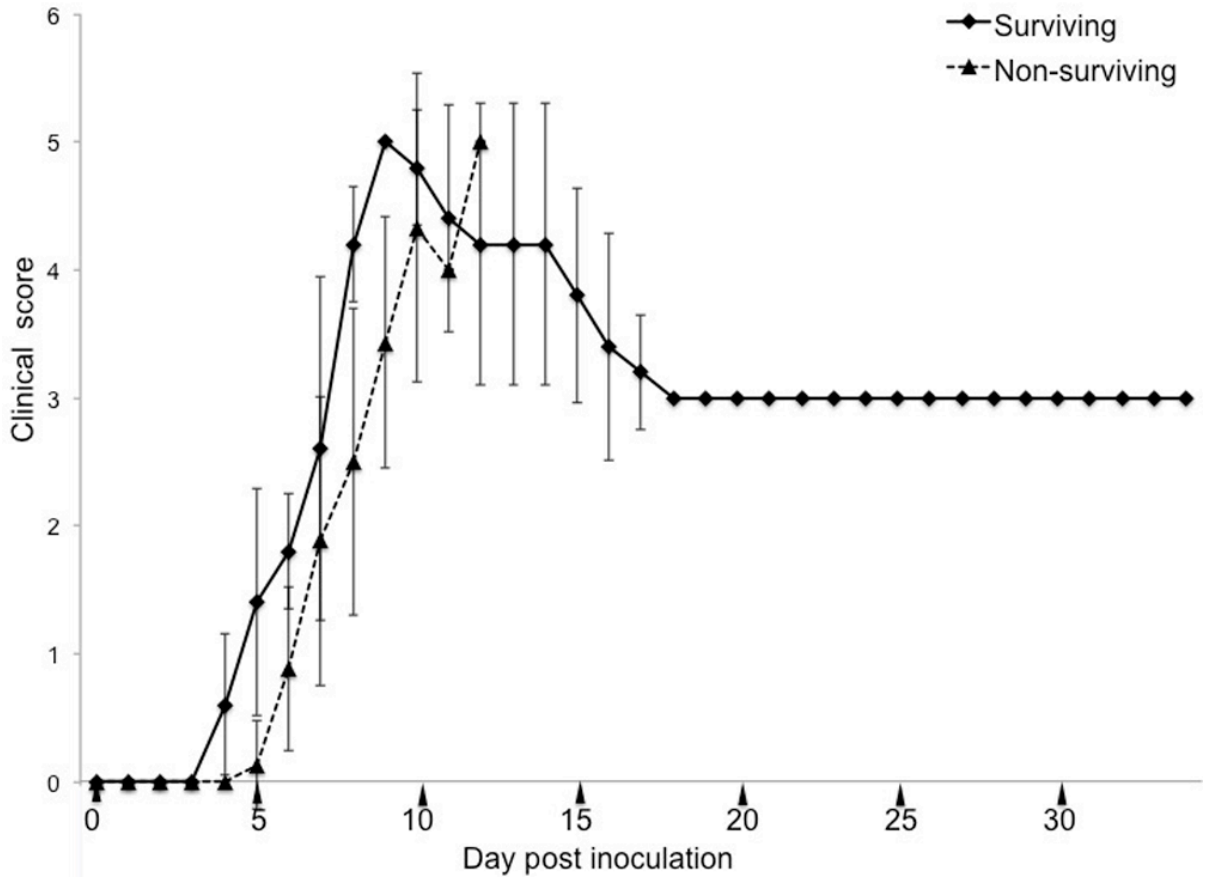

Figure 1.2. Clinical symptom scoring in surviving and non-surviving rabbits. The scores were determined based on the severity of the symptoms in each case with score 0-5 including 0, no symptoms; 1, unstable gait and lack of coordination of the hind limbs; 2, paralysis of the hind limbs; 3, lateral recumbency with ability to drink and eat; 4, lateral recumbency without ability to drink and eat; 5, systemic convulsion with spasm.

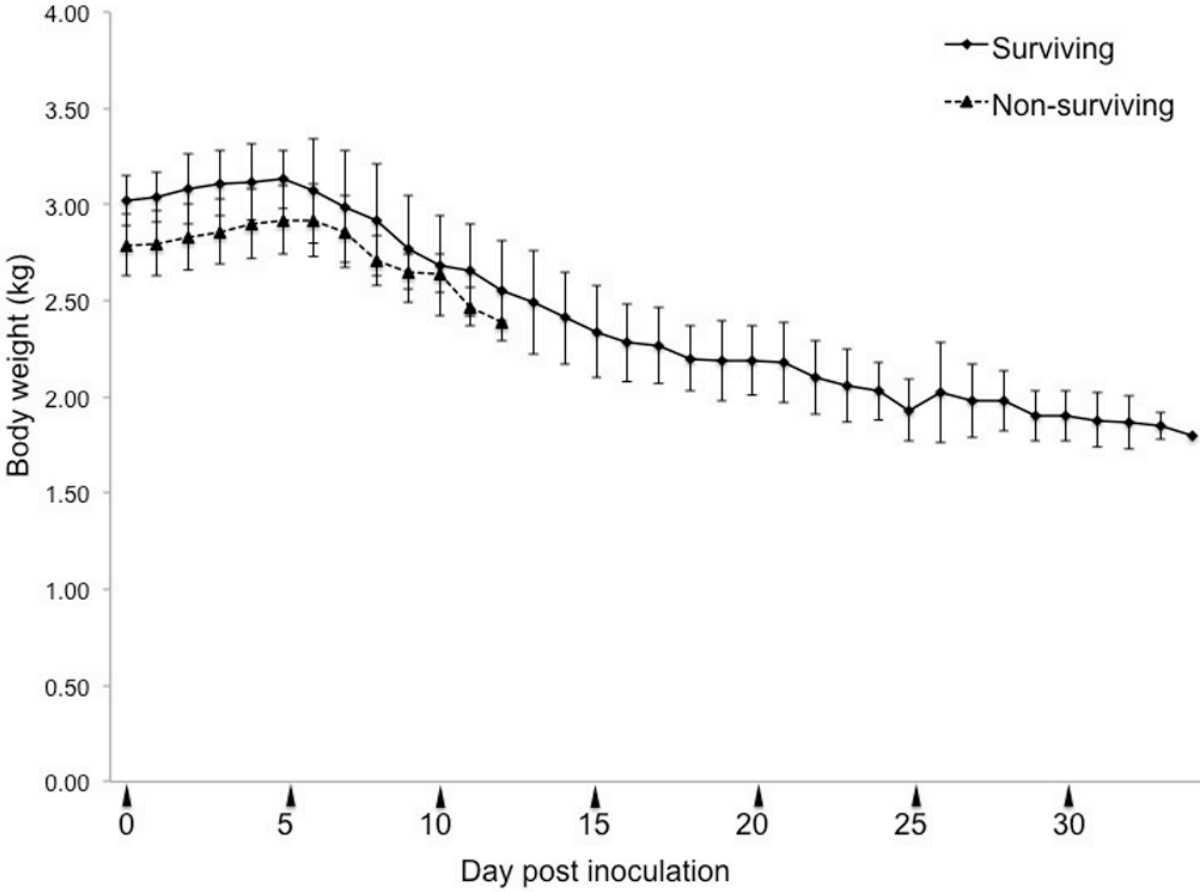

Figure 1.3. Body weight changes in surviving and non-surviving rabbits. The weight of surviving rabbits gradually decreased even after the peak of rabies symptoms around 10 dpi to the end of experiment.

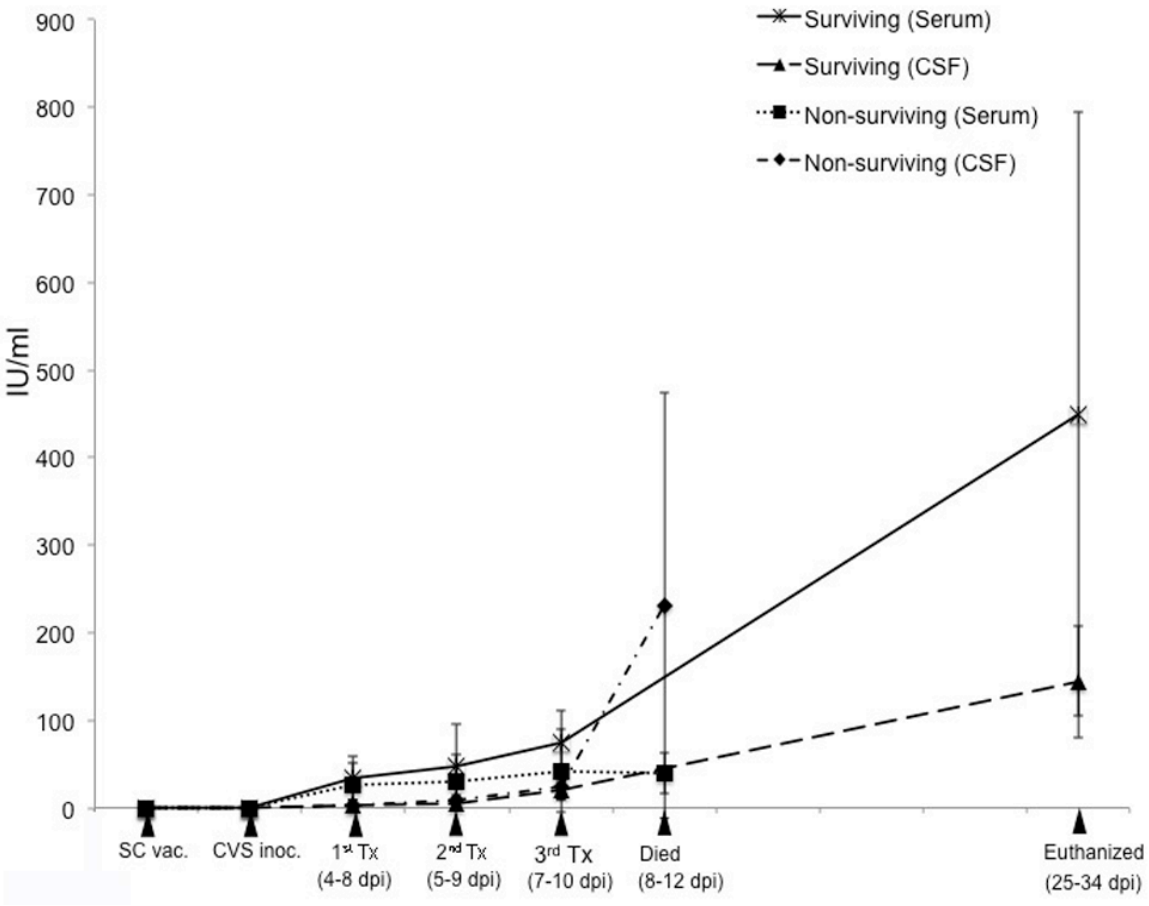

Figure 1.4. Viral neutralizing antibody titers in the serum and CSF. The serum and CSF were collected at each time point as Figure 1.1 including SC vaccination, CVS inoculation, 1st treatment (4-8 dpi), 2nd treatment (5-9 dpi), 3rd treatment (7-10 dpi), died (8-12 dpi), and euthanasia (25-34 dpi). Tx: treatment.

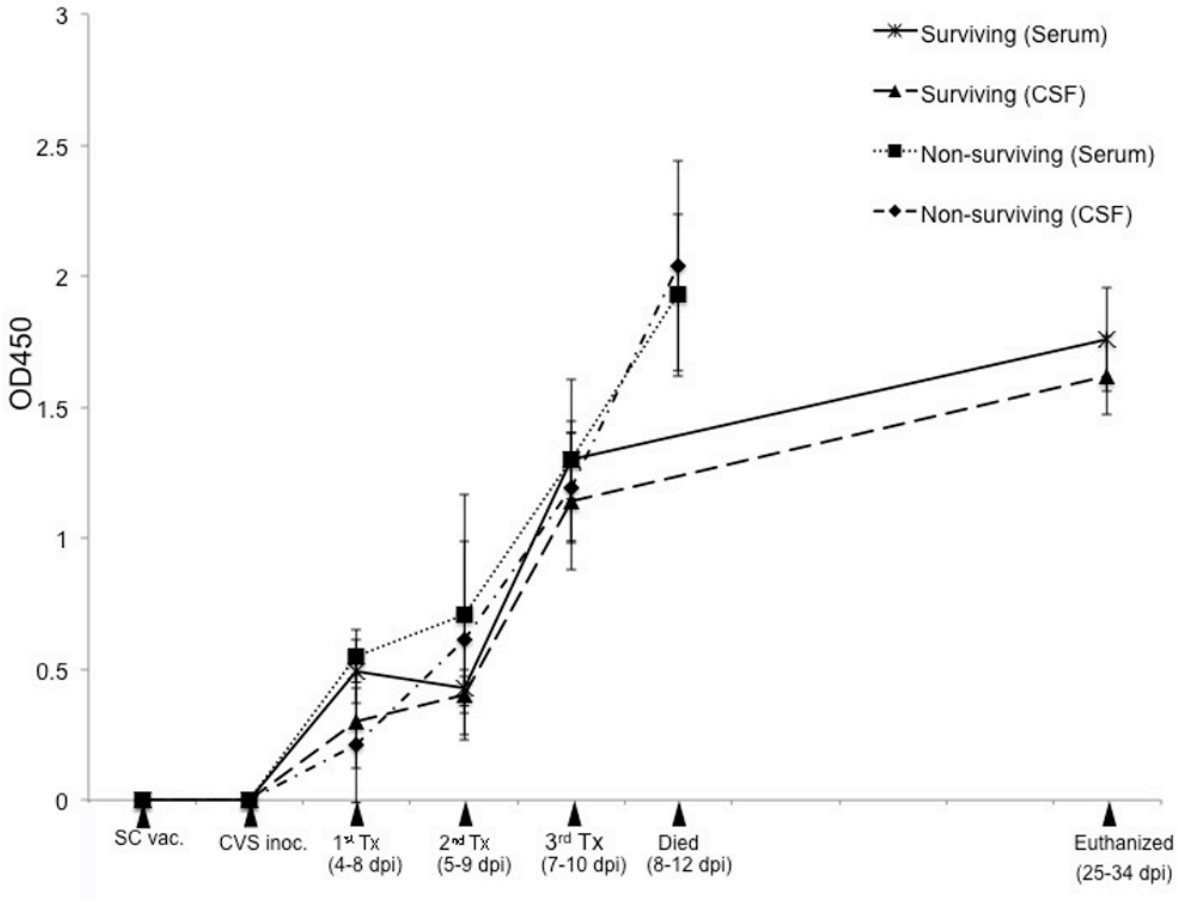

Figure 1.5. ELISA antibody titers in the serum and CSF. The serum and CSF were collected at each time point as Figure 1.1 including SC vaccination, CVS inoculation, 1st treatment (4-8 dpi), 2nd treatment (5-9 dpi), 3rd treatment (7-10 dpi), died (8-12 dpi), and euthanasia (25-34 dpi). Tx: treatment.

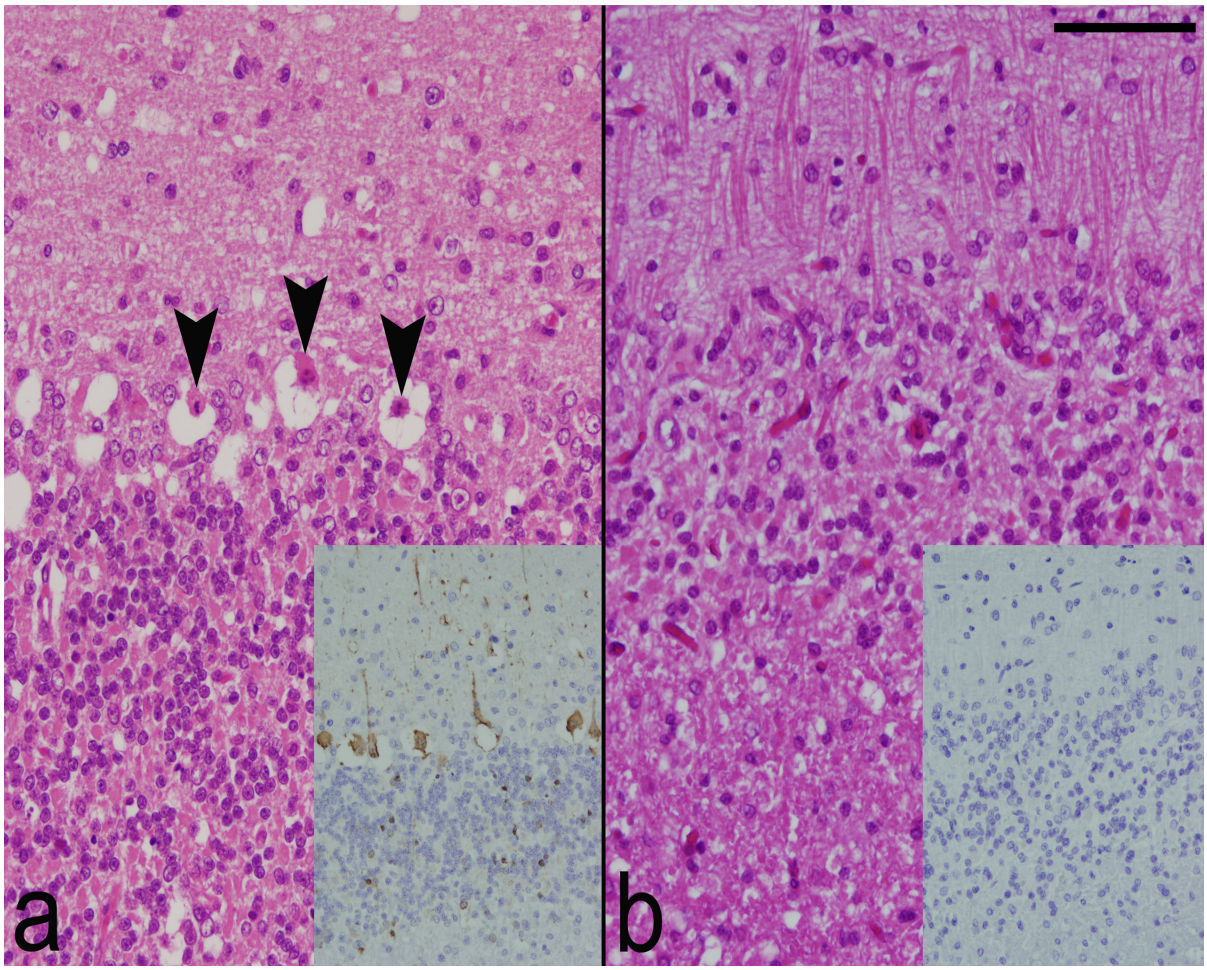

Figure 1.6. The cerebellar cortex of a non-surviving rabbit from the non-treatment group (a) showing necrosis of Purkinje cells (arrowhead). Immunohistochemistry of the cerebellum reveals abundant RABV antigen in Purkinje and granular cells (inset, a). The cerebellar cortex of a surviving rabbit from the SC/IT group b) shows the loss of Purkinje and granular cells together with gliosis of molecular layer. RABV antigen is not found in the cerebellum (inset, b). Scale bar represents 50 μm.

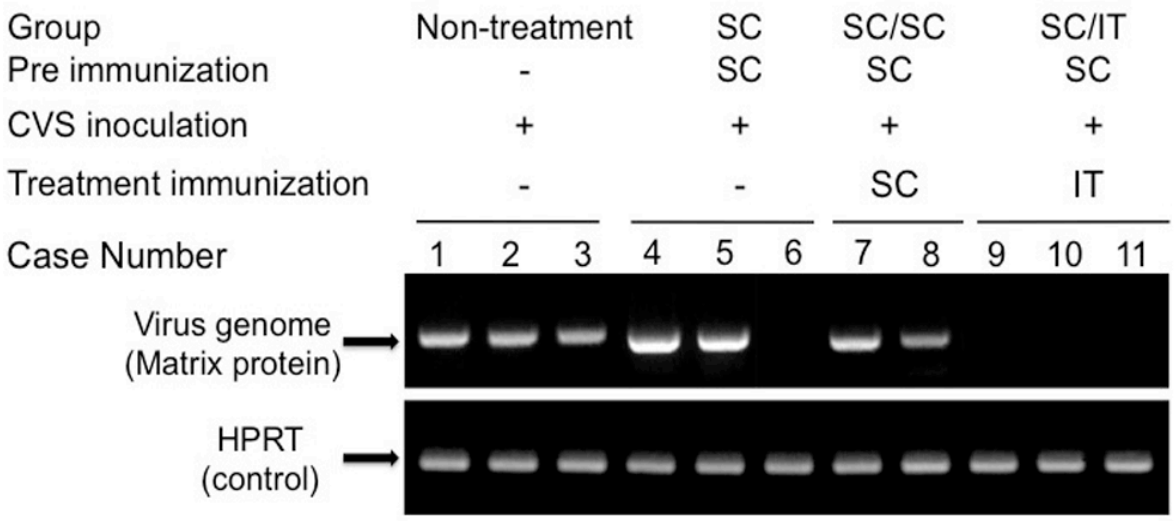

Figure 1.7. A positive signal of the RABV matrix gene were detected in the brain samples from all rabbits except one surviving rabbit in the SC group (lane 6) and three surviving rabbits in the SC/IT group (lanes 9-11) by RT-PCR. HPRT; Hypoxanthine phosphoribosyltransferase was used as an internal control.

Table 1. Summary of the clinical course of the rabbits of four groups

Groups	ID	Neuromuscular symptom			Terminal point (dpi)	Euthanized (dpi)	Lethality (%)
		Appearance (dpi*)	Peak (dpi)	Recovery (dpi)			
Non-treatment	1	6	9	-	12	-	100
	2	8	10	-	12	-	
	3	7	9	-	10	-	
SC	1	6	9	-	10	-	66.7
	2	5	8	-	8	-	
	3	4	9	16	-	32	
SC/SC	1	6	8	-	10	-	100
	2	6	10	-	10	-	
	3	6	9	-	9	-	
SC/IT	1	4	7	11	-	25	0
	2	6	8	18	-	34	
	3	5	7	12	-	33	
	4	4	9	16	-	32	

* dpi: days post inoculation

Chapter 2

Neurogenic cardiomyopathy in rabbits with experimentally induced rabies

Introduction

Domestic rabbits fed a high-fat diet develop inducible hypercholesterolemia and vascular lesions, which makes them a suitable model for cardiovascular research (Pariaut, 2009). Cardiomyopathy has been rarely reported in the domestic rabbit (Pariaut, 2009). Anesthesia with a combination of xylazine and ketamine has been reported as a cause of cardiovascular lesions in Dutch Belted rabbits (Hurley et al., 1994). In humans, the interaction between the brain and heart has been a focus of much research (Feiden, 2013; Guglin and Novotoraova, 2011; Mashaly and Provencio, 2008; Meyer et al., 2009), and brain lesions, particularly subarachnoid hemorrhages, have been shown to be a crucial contributing factor for myocardial damage (Feiden, 2013; Guglin and Novotoraova, 2011; Mashaly and Provencio, 2008; Meyer et al., 2009). In this study, I describe neurogenic cardiomyopathy in rabbits with experimentally induced rabies.

Materials and Methods

Virus

Two strains of RABV were selected to use in this study including the challenge virus standard (CVS) strain and street strain of RABV (strain 1088).

Animal and Experimental design

A total of 18 New Zealand White rabbits (16-week-old, Japan SLC Inc., Japan), weighing 2.92 ± 0.18 kg (mean±SD), were used in this study. Fourteen rabbits were intramuscularly inoculated in the hind leg with 2 mls of the CVS strain of RABV at a dose of 4×10^7 focus forming unit (FFU)/ml. The remaining four rabbits were inoculated in the masseter muscle or

nasal mucosa with 0.2 mls of street RABV (strain 1088) at 5 x 10^6 FFU/ml.

The rabbits were observed daily for symptoms of rabies. The rabbits were anesthetized 4–6 times using xylazine hydrochloride (2 mg/kg Selactar; Bayer Health Care, Germany) and ketamine hydrochloride (35 mg/kg Ketalar; Daiichi Sankyo Co., Japan) in order to collect peripheral blood and cerebrospinal fluid. The frequency of xylazine/ketamine anesthesia for each rabbit is summarized in Table 2.1.

Histopathology and immunohistochemistry

All rabbits were necropsied and heart and brain samples were collected and fixed in 20% buffered formalin. The fixed tissues were embedded in paraffin wax and cut into 4 μm sections for routine hematoxylin and eosin (HE) staining. The heart sections were also stained with Masson trichrome. Immunohistochemistry (IHC) was performed for both brain and heart sections using a streptavidin-biotin-peroxidase system (SAB-PO kit; Nichirei Bioscience, Japan). Monoclonal mouse anti-rabies nucleoprotein antibody (clone N13-27; kindly provided by Dr. Naoto Ito, Gifu University) was used as the primary antibody. The protocol was in accordance that described in Chapter 1.

Ethic statement

These animal experiments were carried out in accordance with the Animal Care and Use Committee of Hokkaido University (approval number 09-0028) and Oita University (approval number M010002).

Results

Thirteen of the 14 rabbits inoculated with the CVS strain of RABV showed signs of rabies, including unstable gait of the hind limbs and decreased food and water consumption, within 8 days post inoculation (dpi). The rabbits progressively deteriorated and exhibited systemic convulsions by 10 dpi. The remaining rabbit showed no clinical signs. Eight of the 13 rabbits

died within 3–6 days after first showing rabies symptoms. The other five rabbits recovered and survived until the end of experimental period (25–34 dpi). All the rabbits inoculated with street RABV showed the transient clinical symptom of decreased food and water consumption within 9–12 dpi and returned to normal within 4–5 days after that.

Macroscopically, the two rabbits that survived CVS challenge showed multifocal white streaks in the myocardium of the left ventricle and interventricular septum (Figure 2.1).

Microscopically, the eight rabbits (Nos. 1–8) that died of CVS challenge showed neuronal necrosis with occasional neuronophagia and deposition of a large amount of RABV antigen in the nerve cell bodies/projections throughout the CNS (Figure 2.2). These lesions were most severe in the cerebral cortex, the thalamus, the hypothalamus, the nuclei of ascending pathways and reticular formation of the brain stem, the cerebellar vermis, the dorsal horn and intermediate substance of the gray matter of the spinal cord, and the lumbar and sacral dorsal ganglia. Malacic foci were occasionally found in the medulla. In addition, perivascular infiltration by lymphocytes and proliferation/hypertrophy of microglia were observed in these lesions (data not shown).

In the five surviving rabbits (Nos. 9–13), neuronal necrosis was most obviously seen in the Purkinje cells and granular cells of the cerebellar vermis, the pontine reticular nuclei, and the tegmental areas of the brain stem. Small malacic foci, mild astrogliosis, lymphoplasmacytic meningitis, and perivascular cuffing were also found in these areas. RABV antigen was rarely detected in the brains of these rabbits. No brain lesion or RABV antigen were observed in one rabbit (No. 14) that was challenged with CVS and survived without clinical signs.

In street RABV-inoculated rabbits (Nos. 15–18), mild perivascular infiltration by lymphocytes and plasma cells was seen in the meninges of the brain, but RABV antigen was not detected immunohistochemically in the brains of these rabbits.

Mild to severe, multifocal myocardial necrosis was found in all eight rabbits (Nos. 1–8) that died of CVS challenge and in five (Nos. 9–13) of the six rabbits that survived the challenge, and the degree of the myocardial lesions correlated with the severity of the brain lesions in each rabbit. The rabbit that did not show clinical signs of rabies (No. 14) and the rabbits inoculated with street RAB, had no myocardial lesions (Table 2). The necrotic foci were mainly located in the myocardium of the left ventricle and the interventricular septum, accompanied by various degrees of fibrosis (Figure 2.3) and mild infiltration of mononuclear cells and heterophils. The necrotic myocardial cells showed hyalinized and/or vacuolated cytoplasm, loss of cross striation and occasional cytoplasmic contraction bands (Figure 2.4). None of the rabbits were positive for RABV antigen in the cardiac tissue.

Discussions

After reaching the CNS via the peripheral nerves, street RABV disperses centrifugally to various organs including the heart (Cheetam et al., 1970; Cheung and Hachinski, 2000; Metze and Feiden, 1991), whereas the CVS strain, a neurovirulent fixed RABV strain, does not descend from the CNS to the periphery. Therefore, the CVS strain does not infect myocardium. In previous reports, the combination of xylazine and ketamine used for anesthesia was considered as a possible cause of cardiomyopathy in rabbits (Mashaly and Provencio, 2008), and the vasoconstriction caused by xylazine or xylazine-like agents has been proposed as a mechanism (Hurley et al., 1994). However, in our study, myocardial necrosis appeared independently of the frequency/cumulative dose of the anesthesia. Instead, the myocardial lesions appeared only in the rabbits having brain lesions, and the degree of the brain lesions correlated well with the severity of the myocardial lesions in each rabbit. The brain lesions were most prominent in the cerebral cortex, the thalamus, the hypothalamus and the medulla. In humans, myocardial damage had been commonly reported in patients with

brain lesions, particularly those involving the insular cortex, the hypothalamus, and/or the brain stem (Cheung and Hachinski, 2000; Guglin and Novotoraova, 2011; Meyer et al., 2009). These changes cause the release of catecholamine, which is directly secreted into the heart via the nerves (Mashaly and Provencio, 2008). The myocardial damage caused by brain lesions is characterized by hypereosinophilic and/or vacuolated cytoplasm, loss of cross striation, contraction bands necrosis, fibrosis, and infiltration of inflammatory cells, as seen in this study (Feiden, 2013; Guglin and Novotoraova, 2011; Mashaly and Provencio, 2008; Meyer et al., 2009). Contraction band necrosis in myocardial cells has been previously reported as a marker of the catecholamine surge (Guglin and Novotoraova, 2011). Therefore, the myocardial lesions appeared in the rabid rabbits of the present study were classified as neurogenic cardiomyopathy.

Summary

Cardiomyopathies are rarely described in the rabbits. Here I describe myocardial necrosis of the ventricular wall in rabbits with experimentally induced rabies. Myocardial lesions were found only in rabbits with brain lesions, and the severity of the cardiac lesions was proportional to that of the brain lesions. Neither the frequency nor the cumulative dose of anesthesia was related to the incidence or the severity of the myocardial lesions. The myocardial lesions were characterized by degeneration and/or necrosis and were accompanied by contraction band necrosis, interstitial fibrosis, and infiltration of inflammatory cells. The brain lesions due to rabies virus infection were most prominent in the cerebral cortex, thalamus, hypothalamus, brain stem, and medulla. Rabies virus antigen was not found in the hearts of any rabbits. Based on these findings, the myocardial lesions were classified as neurogenic cardiomyopathy.

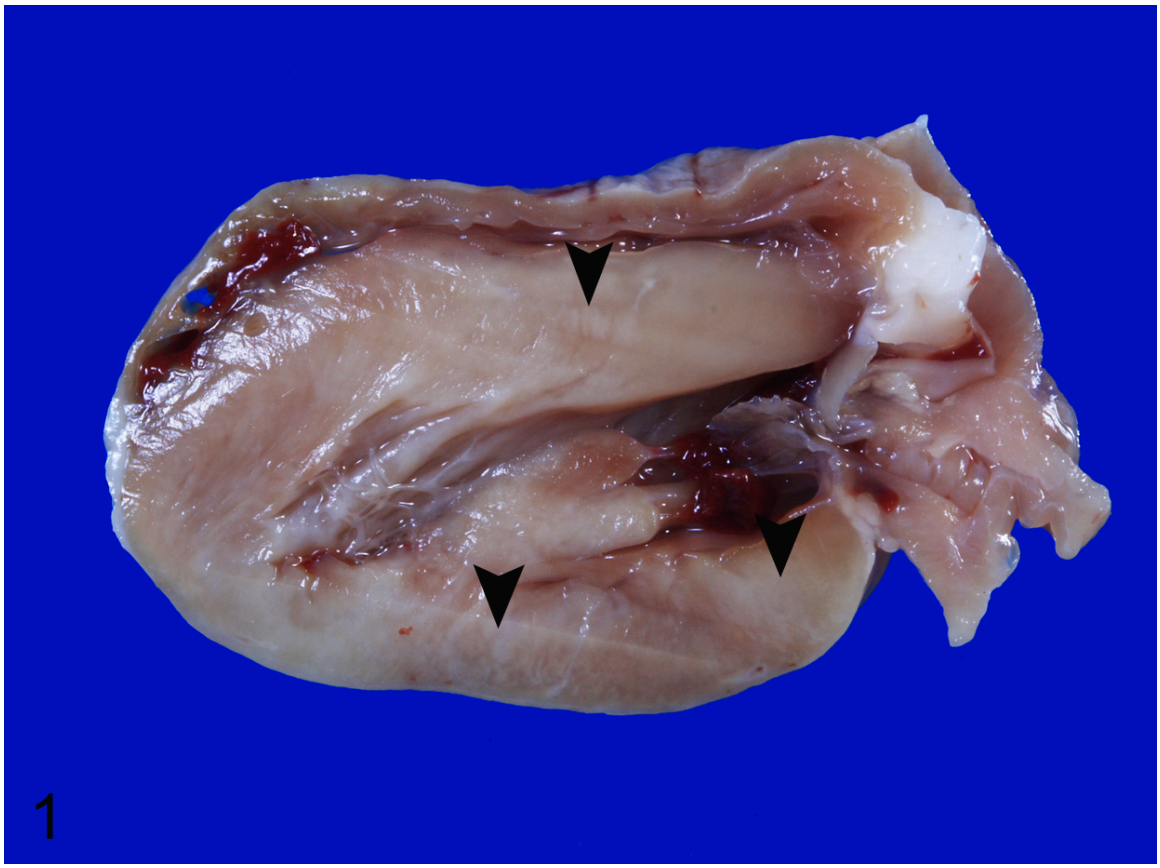

Figure 2.1. Heart, rabbit No. 12, 33 dpi. Formalin-fixed heart specimens revealed multiple white streaks (arrowheads) consisted with myocardial necrosis and fibrosis of the left ventricle and interventricular septum.

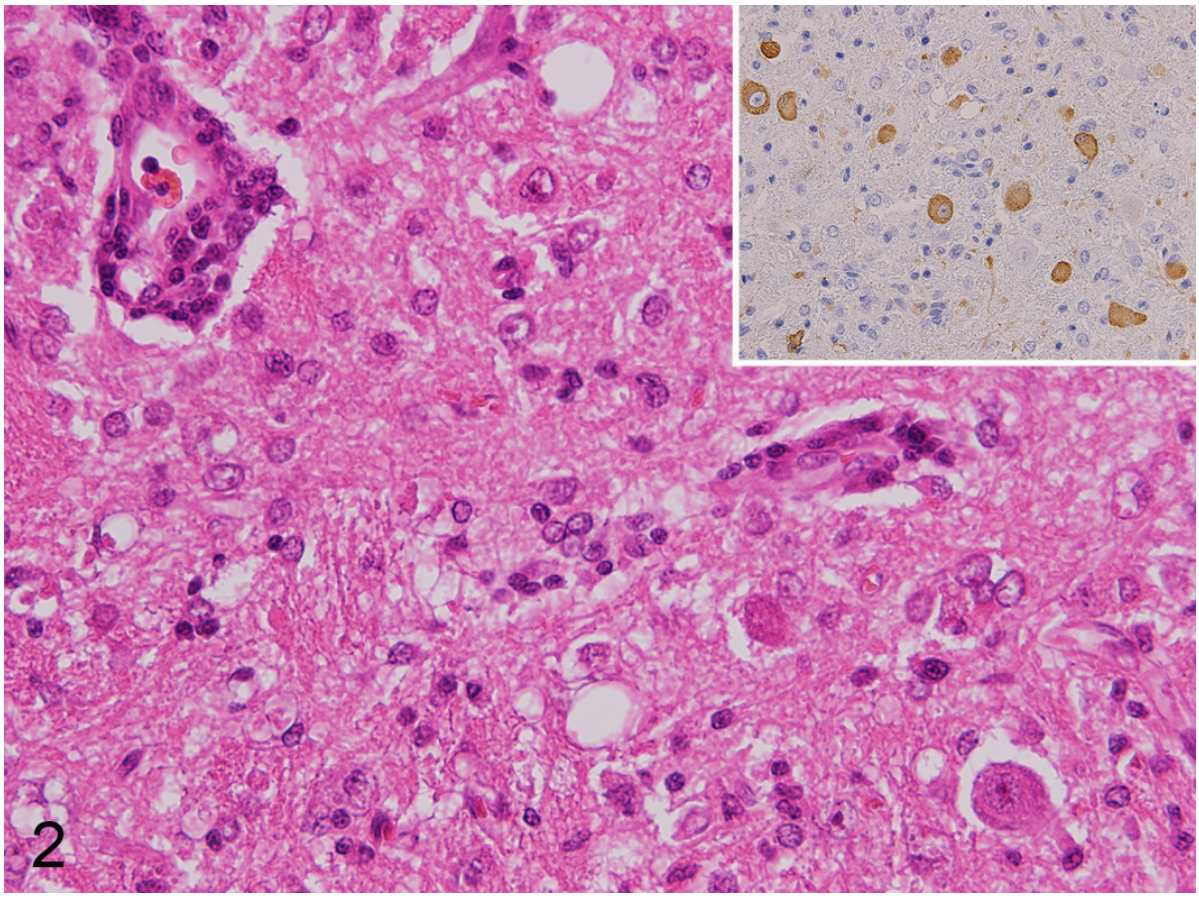

Figure 2.2. Brain stem, rabbit No. 2, 12 dpi. Non-suppurative encephalitis with encephalomalacia is observed at the brain stem. HE. Inset: IHC (SAB-PO kit) with a monoclonal anti-RABV nucleoprotein and counterstaining with Mayer's hematoxylin revealed a large amount of RABV antigen in the cytoplasm and nerve process of the neurons.

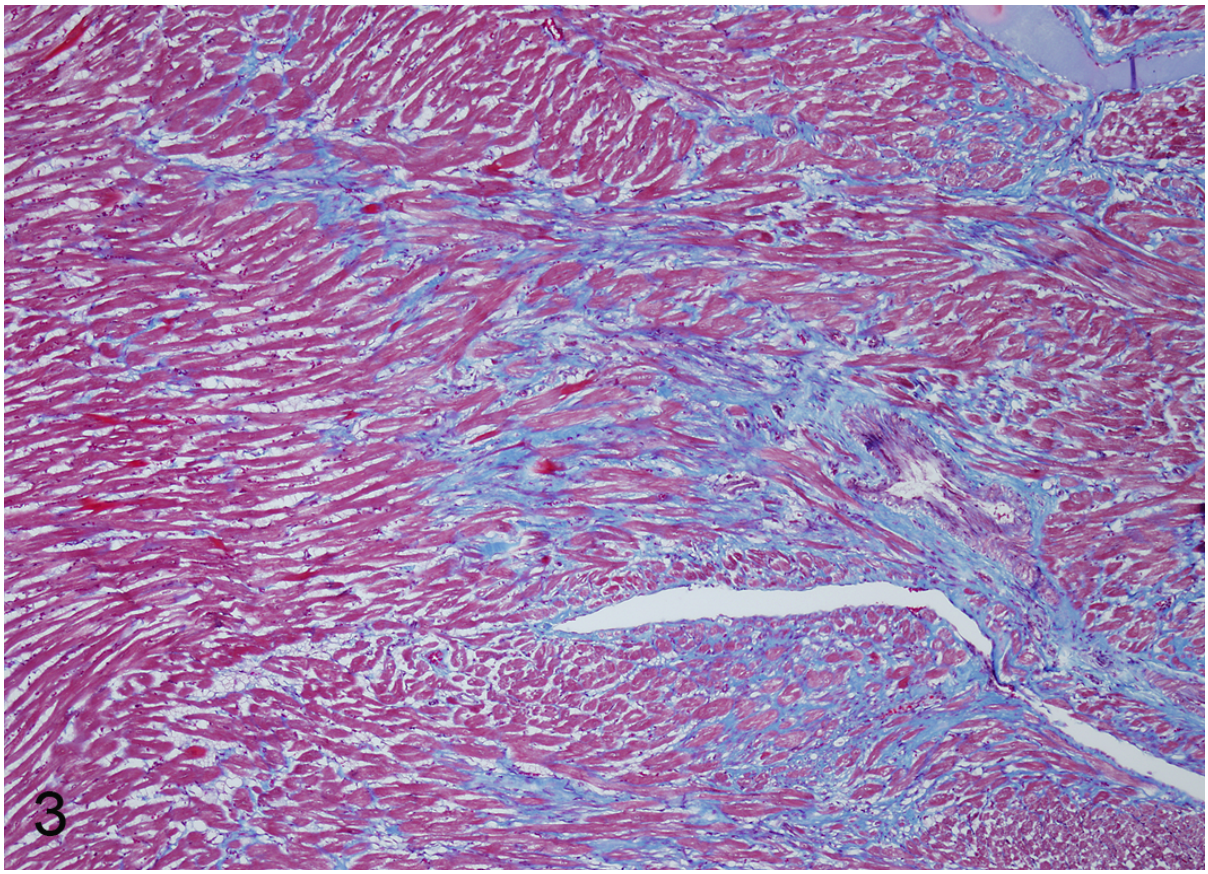

Figure 2.3. Left ventricle, rabbit No. 12, 33 dpi. Diffuse interstitial fibrosis accompanied by distortion of the arrangement of the myocardial cells. Masson's trichrome stain.

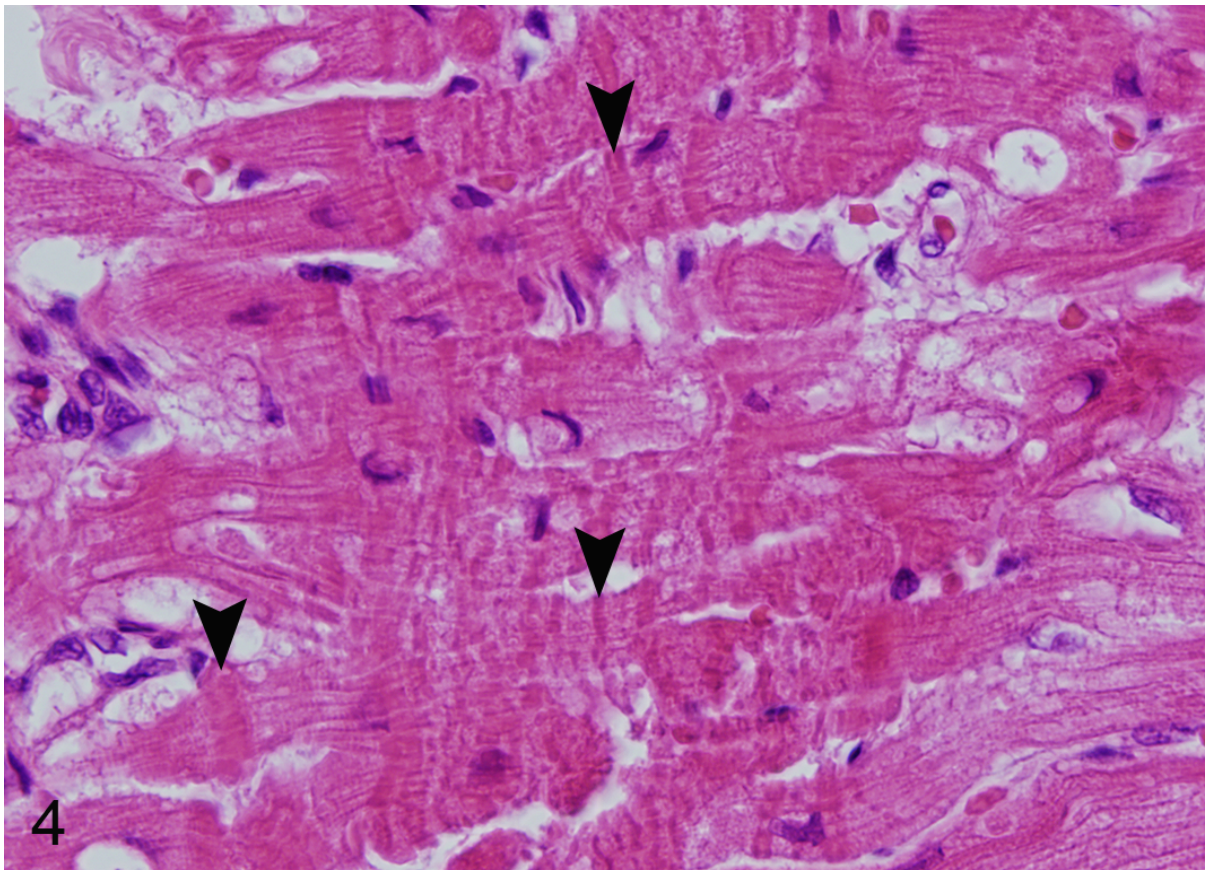

Figure 2.4. Left ventricle, rabbit No. 10, 25 dpi. Myocardial degeneration is characterized by hypereosinophilic and vacuolated cytoplasm and contraction band necrosis (arrowheads). HE.

Table 2. The relationship between heart lesions and the frequency of anesthesia, neurological signs, and brain lesions.

ID	Challenged virus	Outcome	Frequency of anesthesia[#]	Neurological signs*	Neuronal necrosis**	Myocardial lesion**
1	Fixed (CVS strain)	Died	5	+	++	+++
2			5	+	++	++
3			5	+	+++	++
4			4	+	++	+
5			4	+	+	+
6			5	+	++	++
7			5	+	+	+
8			4	+	+	+
9		Survived	6	+	++	+
10			6	+	++	++
11			6	+	++	+++
12			6	+	++	+++
13			6	+	++	+
14			6	-	-	-
15	Street （1088 strain）	Survived	6	-	-	-
16			6	-	-	-
17			6	-	-	-
18			6	-	-	-

[#] Number of ketamine/xylazine administrations; *+: positive, -: none. ** +++: severe, ++: moderate , +: mild, -: intact.

General conclusion

Rabies is a fatal zoonotic disease for which no effective treatment measures are currently available. Previous reports of my laboratory have described that SC immunization prior to IT immunization induces a more rapid and higher antibody response in the CSF than IT immunization alone. The VNA, which is crucial in RABV clearance, is originated both from serum and *de novo* antibody locally produced in the CNS. Therefore, I considered that SC immunization prior to IT immunization might be applicable to rabid animal as a therapeutic measure for rabies.

In the first experiment, 10 rabbits exhibited neuromuscular symptoms of rabies within 4-8 dpi. The rabbits died within 8-12 dpi with the exception of one rabbit in the SC group and all four rabbits in SC/IT group, which recovered and resumed responding external stimuli, eating and drinking by hand feeding at 11-18 dpi and survived until the end of the experimental period. RABV was eliminated from the CNS of the surviving rabbits. It was speculated that humoral and cellular immunities were responsible for RABV clearance from the CNS and recovery. Although my treatment protocol was still incomplete as an appropriate therapy for rabies, the results indicate that IT immunization can at least rescue the life of experimentally induced rabid rabbits and prompt the future development of novel therapies against rabies.

In the second experiment, experimentally induced rabid rabbits, which had brain lesions, showed myocardial necrosis accompanied by contraction band necrosis, which is the marker of catecholamine surge, and the severity of the cardiac lesions was proportional to that of the brain lesions. Rabies virus antigen was not found in the hearts of any rabbits. Neither the frequency nor the cumulative dose of anesthesia was related to the incidence or the severity of the myocardial lesions. Based on these findings, the myocardial necrosis of rabid rabbits was classified as neurogenic cardiomyopathy.

In conclusion, SC/IT immunization protocol described here may be applicable for the treatment of rabies. However, the protocol await further modification for the perfect therapy of rabid patients. The present study also demonstrated that rabid rabbits had myocardial necrosis as a complication of the brain lesions due to RABV infection.

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Summary in Japanese

狂犬病は致死的な人獣共通感染症で、現在もなお有効な治療法がない。本研究室ではこれまでに鞘内免疫の前に皮下免疫を施すと鞘内免疫単独よりも高い抗体価をより迅速に脳脊髄液に誘導すること、脳からの狂犬病ウイルス排除に重要な脳脊髄液中和抗体は末梢血中抗体および中枢神経組織で産生された抗体の２つの起源を持つことを報告してきた。これらの結果に基づき、私は、鞘内免疫を用いて狂犬病を治療できるのではないかと考えた。

第 1 章では、固定毒狂犬病ウイルス接種 4~8 日後に神経筋症状を呈した 10 羽のウサギを用いて実験を行った。皮下免疫のみを行った 3 羽中 1 羽および皮下免疫後鞘内免疫を行った 4 羽中 4 羽はウイルス接種後 11~18 日後から重篤な神経症状から回復し、外部刺激への反応と飲食を再開し、実験終了後まで生残したが、それらの 5 例を除く全例がウイルス接種後 8~12 日以内に斃死した。生残したウサギの中枢神経組織ではウイルスが排除されており、それには液性および細胞性免疫が関与していると推測された。本実験では、完全な回復には至らなかったものの、鞘内免疫によって狂犬病発症個体の延命が可能であることが証明され、この知見は新規狂犬病治療法開発の礎になると思われた。

第 2 章では、固定毒狂犬病ウイルス接種に起因する脳病変を有するウサギは、カテコールアミン過分泌に特徴的な収縮帯壊死を伴う心筋壊死に陥ることを発見し、その発生機序を検討した。各ウサギの心筋病変の程度は脳病変のそれにほぼ比例しており、どの症例においても心筋への狂犬病ウイルス感染は認められなかった。さらに、心筋病変の発生は麻酔薬の投与回数および投与量と無関係であった。これらの所見から、狂犬病罹患ウサギの心筋壊死は神経原性心筋症であると結論された。

結論として、本研究では、鞘内免疫によって狂犬病を治療出来る可能性があるが、現在のプロトコールでは不完全であり、更なる改善を要すること、狂犬病の併発症として神経原性心筋症が発生することを明らかにした。