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**記載語彙**

N�HOKKAIDO UNIVERSITY
Survival of rabid rabbits after intrathecal immunization

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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 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 using the same vaccine, and four rabbits received three intrathecal (IT) immunizations, in which the vaccine was inoculated directly into the cerebrospinal fluid (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. We report here a possible, although still incomplete, therapy for rabies using IT immunization. Our protocol may rescue the life of rabid patients and prompt the future development of novel therapies against rabies.

Key words: animal experiment, CNS pathology, intrathecal immunization, rabies virus, treatment of rabies.

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.1 High levels of viral neutralizing antibody (VNA) have been found in the cerebrospinal fluid (CSF) in rare recovered cases of rabies.2–6 Two rabid human patients survived after being put into a therapeutic coma under the so-called “Milwaukee protocol”.5,6 However, therapeutic coma was not successful in other patients, and the scientific rationale of the Milwaukee protocol remains controversial.3,7

Recent virological studies have revealed that RABV8,9 has anti-apoptotic and anti-inflammatory effects that suppress nerve cell damage and inflammatory responses in the CNS of heavily infected animals.10,11 These features imply that the elimination of RABV from the CNS by appropriate treatment could lead to recovery from rabies.

We have reported that intrathecal (IT) immunization, which involves the direct inoculation of antigens into the CSF, induced specific antibodies against RABV in the CNS,12,13 The immunization induced a protective immune response against the transneural spread of RABV in mice.14 Subcutaneous (SC) immunization prior to IT immunization induced a more rapid and higher antibody response in the CSF than IT immunization alone.12 We 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. Our protocol may rescue the life of rabid patients and prompt the future development of novel therapies against rabies.

**MATERIALS AND METHODS**

**Virus**

The CVS strain, a neurovirulent, fixed RABV\textsuperscript{15} 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.\textsuperscript{15,16} Furthermore and more importantly, the latent period between virus inoculation and the clinical appearance of rabies is fixed at about 1 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., Hamamatsu, Japan) were used in this study. Twenty-four rabbits were injected subcutaneously with 1 mL of the inactivated whole RABV particles (Nisseiken Rabies TC Vaccine, Nisseiken Co., Tokyo, 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\textsuperscript{7} FFU/mL). Ten of the 24 rabbits (41.7\%) showed neuromuscular symptoms of rabies prior to 8 days post-inoculation (dpi) and the remaining 14 rabbits were excluded from the experiment. These 10 rabbits were allocated into three groups. Three rabbits received no further treatment after showing symptoms of rabies (the SC group); three rabbits received three additional SC immunizations (the SC/SC group) using the vaccine and four rabbits were treated with three 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, Leverkusen, Germany) and ketamine hydrochloride (35 mg/kg Ketalar; Daiichi Sankyo Co., Tokyo, Japan). An additional three naïve rabbits were inoculated intramuscularly with RABV and no vaccination was given (the non-treatment group; see Figure 1 for the treatment schema). All the recumbent rabbits were given daily injections of 100–150 mL saline containing 5% glucose and 10 mL of amino acid solution (Aminoleban, Otsuka Pharmaceutical Co., Tokyo, 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 2 and 3 and were stored at −20°C until antibody titers were assayed. The VNA assay was performed using a rapid fluorescent focus inhibition test, as previously described.\textsuperscript{2,17} ELISAs were conducted as previously described.\textsuperscript{12}

**Histopathology and immunohistochemistry**

Selected tissues, including visceral organs and nervous tissues, were collected and fixed in 20% buffered formalin for histopathological examination. For immunohistochemistry (IHC), a streptavidin-biotin-peroxidase system.
(SAB-PO Kit; Nichirei Bioscience, Tokyo, Japan) was employed. Primary antibodies used for IHC were monoclonal mouse anti-rabies nucleoprotein (clone N13-27; kindly provided by Dr. Naoto Ito, Gifu University), monoclonal mouse anti-human GFAP (clone 6F2; DAKO, Carpinteria, CA, USA), monoclonal mouse anti-human CD3 (clone F7.2.38; DAKO, USA), monoclonal mouse anti-human CD79α (clone MH57; DAKO), and goat polyclonal anti-rabbit Iba-1 (code ab5076; Abcam, Cambridge, UK).

RT-PCR
Total RNA was extracted from brain tissue using the RNeasy Kit (Qiagen, Germantown, MD, USA) and 5 μg of RNA was used for reverse transcription with the SuperScript First-Strand Synthesis system (Life Technologies, Carlsbad, CA, USA). The fragment of the RABV genome encoding matrix protein was amplified using Go Taq DNA polymerase (Promega, Madison, WI, 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 $P < 0.05$.

Ethics statement
All animal experiments were conducted within 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 the 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 Fig. S1. 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 (Fig. S1). However, they remained recumbent, with decreased body weight (Fig. S2) 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 (Figs 2, 3). 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
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 pathways and reticular formation of the brain stem, the vermis of the cerebellum (Fig. 4a), 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 meninges, perivascular spaces of the CNS and dorsal ganglia, and T lymphocytes were dominant over B lymphocytes in number at all 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, 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, 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 present in small numbers. RABV antigen was rarely

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

<table>
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<tr>
<th>Groups</th>
<th>ID</th>
<th>Appearance (dpi)</th>
<th>Peak (dpi)</th>
<th>Recovery (dpi)</th>
<th>Terminal point (dpi)</th>
<th>Euthanized (dpi)</th>
<th>Lethality (%)</th>
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<tr>
<td>Non-treatment</td>
<td>1</td>
<td>6</td>
<td>9</td>
<td>–</td>
<td>12</td>
<td>–</td>
<td>100</td>
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<tr>
<td></td>
<td>2</td>
<td>8</td>
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<td>–</td>
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<td></td>
<td>3</td>
<td>7</td>
<td>9</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>66.7</td>
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<tr>
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<td>1</td>
<td>6</td>
<td>9</td>
<td>–</td>
<td>10</td>
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<td>9</td>
<td>16</td>
<td>–</td>
<td>32</td>
<td>–</td>
</tr>
<tr>
<td>SC/SC</td>
<td>1</td>
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<td>8</td>
<td>–</td>
<td>10</td>
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<td>100</td>
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<tr>
<td>SC/IT</td>
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dpi, days post inoculation; SC, subcutaneous; IT, intrathecal.

Fig. 4  The cerebellar cortex of a non-surviving rabbit from the non-treatment group showing necrosis of Purkinje cells (arrowheads). Immunohistochemistry of the cerebellum reveals abundant rabies virus (RABV) antigen in Purkinje and granular cells (inset, A). The cerebellar cortex of a surviving rabbit from the subcutaneous (SC)/intrathecal (IT) group showed the loss of Purkinje and granular cells together with gliosis of the molecular layer. RABV antigen is not found in the cerebellum (inset, B). Scale bar represents 50 μm.
detected either in the CNS (Fig. 4b) 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 (Fig. 5).

**DISCUSSION**

The significance of high VNA titers in the CSF has been emphasized in humans and animals after recovery from rabies\(^2\),\(^6\),\(^18\) and VNA is considered as a crucial factor for recovery.\(^18\)-\(^22\) We have reported that IT immunization induced specific antibodies against RABV in the CSF and a protective immune response against the transneural spread of RABV.\(^12\),\(^13\) The immunization could suppress the spread of intracerebrally inoculated RABV in mice.\(^14\) SC immunization prior to IT immunization induced a more rapid and higher antibody response in the CSF than IT immunization alone.\(^12\) Based on these previous findings, we 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 did not were not significantly different at the peak of clinical symptom (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.\(^23\) IT immunization also increases the permeability of the blood-brain barrier (BBB) and allows for the migration of effector cells into the CNS.\(^24\) The B lymphocytes infiltrating the CNS via up-regulation of the chemokine CXCL12\(^25\) are the source of locally produced antibodies important in the clearance RABV from the CNS.\(^2\),\(^12\),\(^20\),\(^26\) In addition, effector T lymphocytes infiltrating the CNS permit the clearance of RABV by inducing apoptosis of infected neurons in the presence of antibodies.\(^27\) These previous reports suggest that a combination of both humoral and cellular immunities\(^17\),\(^19\),\(^24\),\(^28\) contributed to the clearance of RABV from the CNS in the present study.

Our 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 who 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\(^a\) to secure efficient time. However, the success of this therapy was limited because: (i) the damage to the CNS tissue of surviving rabbits was too severe to allow complete recovery; and (ii) a neurovirulent, fixed RABV was used instead of street RABV. Most humans who survived rabies showed severe neural complications after recovery\(^4\) and prompt initiation of IT immunizations may benefit recovery from rabbits with mild neurological complications. The CVS strain causes G protein to be expressed on the surface of infected neurons,\(^16\),\(^29\) which induces apoptosis of the infected neurons and neighboring cells, as well as inflammation in the infected brain.\(^12\),\(^15\),\(^16\),\(^27\),\(^29\) On the other hand, street RABV induces low levels of G protein on the surface of infected neurons due to post-transcriptional modification.\(^11\) The anti-apoptotic, anti-inflammatory and immunosuppressive effects of street RABV infection\(^12\),\(^13\) allow the virus to infect neurons without cellular destruction or inflammation.\(^8\),\(^30\) These findings indicate that the elimination of street RABV from the CNS by IT immunization may lead to a more complete recovery from RABV-induced rabies than from that caused by the CVS strain.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1** 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.

**Fig. S2** Body weight changes in surviving and non-surviving rabbits. The weight of surviving rabbits gradually decreased even after the peak of rabies symptom to the end of experiment.