Effective Prevention against Rabies by Intracerebral Immunization in Mice

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ABSTRACT. To evaluate the efficacy of intracerebral (IC) immunization, mice were immunized with a rabies vaccine by the subcutaneous (SC), intramuscular (IM) or IC route, and 10-fold the 50% lethal dose of rabies virus was inoculated into the hindleg of the immunized or non-immunized mice. The antibody titer in serum was elevated and boosted by additional immunization via all routes, but highest after the IC immunization followed by the IM and SC routes, in this order. Intracerebrally immunized mice were completely protected from death and the neurological signs of infection, whereas the IM or SC immunization only partly protected the mice. In mouse models, IC immunization is more effective at inducing a protective immune response against the transneural spread of rabies virus than IM or SC immunization.

KEY WORDS: intracerebral immunization, mouse, rabies.

Rabies causes a fatal illness with encephalopathy and generalized paresis in humans and animals, and is still a huge public health problem in Asia, Africa and Latin America and reemerging in North America by bat rabies [18, 22, 27]. Rabies virus is a single strand negative sense RNA virus belonging to the Lyssavirus genus of the Rhabdoviridae family [40]. The transmission of rabies virus occurs mainly by contact with the saliva of a rabid animal [17].

Many neurotropic infectious agents including rabies virus [11], herpes viruses [23, 30], corona viruses [25], Bornavirus [14], enterovirus [15] and influenza virus [20, 21, 24, 31] invade the central nervous system (CNS) from the periphery via transneural spread. The protective effect of immunization via ordinary routes is limited for the transneural spread of viruses, since it may result from humoral antibodies that simply neutralize the viruses before they infect nerve cells [37–39]. Thus to obtain better effects against the neurotropic viruses, it is important to block transmission and/or transport in the nervous systems.

In our previous study [30], complete protection against a pseudorabies virus (PRV) challenge was achieved in mice by intracerebral (IC) immunization with inactivated PRV, though the protection was incomplete in subcutaneously immunized mice. In this report, we demonstrate complete protection of mice from rabies virus by IC immunization, and incomplete protection by subcutaneous (SC) and intramuscular (IM) immunization. Possible mechanisms for the complete protection are discussed.

MATERIALS AND METHODS

Virus: Rabies virus (CVS strain) was obtained from Gifu University. For propagation, the virus was inoculated into the brain of a 3-week-old mouse, collected after the animal showed neurological signs, and homogenized with phosphate buffer solution (PBS, pH 7.2). The homogenate was centrifuged for 30 min at 7,000 rpm. The supernatants were collected and stored at –80°C until further use.

Immunization: Forty 4-week-old female ICR mice were purchased from Nippon SLIC Inc. (Shizuoka, Japan) and divided into 4 groups (n=10): negative control (without immunization), SC immunization, IM immunization, and IC immunization. For the SC immunization, 30 µl of inactivated tissue-cultured vaccine (RC-HL strain, Niseiken Co., Japan) was inoculated into the dorsal area of the mice. For the IM immunization, 30 µl of the same vaccine was inoculated into the foreleg. For the IC immunization, 30 µl of the vaccine was inoculated carefully into the right frontal part of the cerebrum using two-step syringe needles (Top Co., Japan) under light anesthesia with ether to maintain the depth of the needle in the brain. Immunizations were performed twice at 2-week intervals with the same method.

Viral challenge: Two weeks after the second immunization, all the mice of each group were infected with 10-fold the 50% lethal dose of rabies virus in the quadriceps muscles of both hindlegs. Survival rates and clinical signs including CNS symptoms were recorded for 15 days after the infection. We necropsied all the dead animals and all animals alive at 15 days post infection (dpi). Half of the organs sampled were processed for histological examination and the other half were frozen at –80°C until homogenized for the recovery of the virus. All animal experiments were carried out with approval of the committee of Laboratory Animal Experimentation, Graduate School of Veterinary Medicine.
Hokkaido University.

Serum antibody titration: Antibody titers in the serum were measured for rabies virus using the rapid fluorescent focus inhibition test (RFFIT) [32, 33]. Two weeks after each vaccination, blood was collected from a tail vein and allowed to clot. Serum was separated by centrifugation (3,000 rpm, 20 min) and stored at −80°C until further use. The results of minimum positive rabies virus neutralization assays were defined as the neutralization of approximately 50-fold the 50% focus-forming dose (FFD50) per 0.1 ml of rabies virus at an initial serum dilution of 1:5 or higher. Once antibodies were detected, the serum was subsequently diluted five-fold. Serum samples were considered negative if no neutralization was observed at a serum dilution of less than 1:5. Results obtained in the RFFIT were transformed to geometric mean titers (GMT). The calculated GMT was compared between groups using a one-tailed analysis of variance (ANOVA, p<0.05).

Histopathology and immunohistochemistry: The liver, spleen, kidneys, heart, lungs, brain (including olfactory bulbs), trigeminal ganglion (TG), spinal cord, dorsal root ganglion (DRG), and hindleg with sciatic nerves were collected and fixed in 20% phosphate-buffered formalin (pH 7.2). The vertebrae and hindleg were decalcified in a 8% formic acid solution after being fixed. The organs were sectioned 4 μm thick and stained with hematoxylin and eosin for light microscopy. For the detection of rabies viral antigens in the tissues, all the sections were stained by the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO kit, Nichirei, Japan) using rabbit anti-rabies virus serum (gift from Drs. Minamoto and Ito, Gifu Univ.) and counterstained with Mayer's hematoxylin.

Viral recovery: To determine the titers of virus in the brains of infected mice, brains were homogenized with autoclaved sea sand and a 10-fold volume of PBS (ml/g of organ), and centrifuged. The supernatant stored at −80°C until titration was serially diluted and inoculated into confluent neuroblastoma (NA) cells in 96-well plates. After incubation at 37°C for 1 hr in a CO2 incubator, the inoculum was discarded, and standard medium was added. The plates were kept for 3 days in a CO2 incubator at 37°C, and the viral titers were determined by FFD50.

RESULTS

Antibody response: The serum antibody titers in the immunized mice of the SC, IM and IC groups are summarized in Fig. 1. In the serum, the titers were elevated in all groups, and boosted by the additional immunization. At each sampling point, the titers of IC immunized mice were the highest, followed by those of IM and SC immunized mice, in this order.

Clinical symptoms and death: The mice showed decreased motor activity right after the IC immunization, but soon recovered and did not show any clinical signs related to the IC immunization thereafter. None of the mice showed clinical signs related to or died from the rabies virus infection until 5 dpi. The first symptoms after infection with rabies virus appeared at 6 dpi in the non-immunization group; mice were anorectic and apathetic, and had decreased motor activity. When these mice were bounded on floor, they did not maintain the normal posture. Later, they showed ataxia and an abnormal gait resembling seals in severe cases, suggesting paralysis of the hindlimbs. After showing lethargy, all the animals died within 4 days. Clinical signs observed in the mice of the SC or IM group were similar to those of non-immunized mice. All the dead animals showed severe emaciation and ruffled hair.

The incidence of neurological signs and survival rates are shown in Fig. 2. There were significant differences in both parameters among the non-immunized mice and three immunized groups. The mice in the non-immunized group began to die from 9 dpi and all had died before 12 dpi when challenged with rabies virus. Thirty percent of intramuscularly immunized mice and 70% of subcutaneously immunized mice showed neurological signs followed by death, after the challenge with rabies virus from 10 dpi. In contrast, none of the intracerebrally immunized mice died or showed neurological signs after being challenged with the virus.

Histopathology and immunohistochemistry: No histological abnormalities were found in visceral organs of any mouse. In the mice that died after the challenge, there was degeneration and necrosis of nerve and glial cells in the DRG, spinal cord, TG, and brain (Fig. 3). Some dead mice had nuclear pyknosis in nerve cells of the pons, cerebellum, cerebrum, ganglia and spinal cord. Viral antigen appeared in all the dead mice, but was not found in the mice that survived until 15 dpi irrespective of immunization and immunization route. In the immunohistochemical slides of dead mice, viral antigens appeared in the nerve and glial cells of the sciatic nerves, DRG, spinal cord and brain stem (pons), hippocampus, cerebellum, cerebrum, TG, and neomuscu-
lar spindles (Fig. 3). We could not find any inflammatory reactions, gliosis, or needle marks in the brain of any of the intracerebrally immunized mice.

**Viral recovery:** To confirm the immunohistochemical findings, the viral titers in the brains of the mice were examined (Table 1). In the dead mice of the non-immunization, SC immunization or IM immunization group, the virus was recovered from the brain and the titer of each group was not significantly different from that of the other groups. Virus was not detected in mice that survived including intracerebrally immunized mice.

**DISCUSSION**

The present study showed that IC immunization using the rabies vaccine yielded greater systemic immune responses than IM or SC immunization, conventional extracerebral routes. Classical theories have emphasized the isolation of the brain from the immune system, and immunological reactions against transneural spread may not be induced before the virus triggers a series of inflammatory reactions initiated by cell death and increased permeability of vascular walls [2]. The absence of a conventional lymphatic system and the presence of the blood-brain barrier are considered the reasons why the CNS is an immunologically privileged site where allogeneic skin of neural grafts that are rejected at conventional extracerebral sites survive in the brain [2]. But, recent reports have shown that immunization via the brain or cerebrospinal fluid (CSF) elicits a systemic humoral immune response, with production of antigen-specific antibodies in the cervical lymph nodes, spleen, serum and CSF [13, 16, 28]. Furthermore, intrathecal (IT) immunization via the subarachnoid (SA) or IC route yields a greater antibody response in the serum or cerebrospinal fluid (CSF) than extracerebral inoculation for a variety of inert antigens, including bacterial toxin and xenogenic albumin, and greater immunogenicity to CSF-administered antigen has been observed under a wide variety of conditions irrespective of whether the brain-blood barrier was disrupted or not [7, 13, 16, 23, 28, 30]. Thus, the enhanced immunogenicity to CSF-administered antigen in this study was considered a normal characteristic of brain/immune system interactions, and these findings strongly suggest that the enhanced response in CSF depends on events initiated within the CNS, and IT production of antibody is required to account for the increase in the level of specific antibody in the CNS following the delivery of antigen to the brain. Theoretically, CSF antibodies may be derived from three sources: passive influx from plasma into the CNS across the blood-brain barrier, carrier-mediated influx across the barrier, or antibody-secreting cells located within the CNS. We previously observed the remarkable increase of antibody titer in the CSF in the rabbits immunized with inactivated influenza virus via the SA [29]. However, there was no significant change in mice immunized via the SC route, which suggests IT synthesis to be the most likely source of the excess
immunoglobulin. Antibody production in the CSF has been reported in normal brain [6] as well as in animals and humans with increased blood-brain barrier permeability or other pathologies of the CNS [1, 10, 34–36]. Immunologic processing with drainage and retention of brain-derived antigens is an important factor in the initiation and regulation of immune responses in the CNS. There are several reports about the efflux of these antigens into peripheral lymphoid organs and their ability to initiate a significant humoral response [6, 7, 13]. Intracerebrally injected soluble protein antigens not only drain into the cervical lymph nodes and initiate antigen-specific immune responses, but also are retained by antigen-presenting cells processed locally and distributed preferentially in the brain parenchyma, resulting in the recruitment of antigen-specific T cells into the brain [19].

Rabies virus infects the CNS by invading neurons in the periphery and then by replicating and spreading to the CNS via synaptically linked neurons in the retrograde direction. In our experiment, the viral antigens were detected in the dorsal root ganglia, spinal cord, pons, cerebrum, cerebellum, trigeminal ganglia, and neuromuscular spindles. These findings indicate that the virus, which was inoculated into the hindleg, reached the brain in the retrograde direction, and subsequently transferred to the trigeminal ganglia and neuromuscular spindles in the anterograde direction. As to the route of viral invasion of peripheral nervous system (PNS), some workers insist that replication of the virus in the muscles is necessary before the virus can enter the PNS [4, 5], whereas others allege direct viral entry into the PNS without replication in muscles [3, 12]. Whether viral replication in the muscle is essential for neuroinvasion or not, viral entry into the peripheral nerves of the hindleg was the important pathway to the CNS, and blocking entry from the inoculated tissues into the nerves by a specific antibody might have caused the decrease in death and neurological signs in the immunized mice in the present study.

In the present study, the IC immunization completely protected mice from death and neurological diseases due to rabies virus infection, while the SC or IM immunization protected the mice only partially. For complete protection by IC immunization, blocking mechanisms other than neu-

Table 1. Recovery of virus in the brain of mice infected with rabies virus after immunization

<table>
<thead>
<tr>
<th></th>
<th>Non-immunization</th>
<th>Subcutaneous immunization</th>
<th>Intramuscular immunization</th>
<th>Intracerebral immunization</th>
</tr>
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<tbody>
<tr>
<td>Dead mice</td>
<td>10/10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>7/7</td>
<td>3/3</td>
<td>10&lt;sup&gt;7.5&lt;/sup&gt;(10&lt;sup&gt;6.5–8.5&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Survived mice</td>
<td>0/3</td>
<td>0/7</td>
<td>0/7</td>
<td>0/10</td>
</tr>
</tbody>
</table>

a) No. of positive mouse for virus recovery / No. of mice examined.
b) Mean titers in organs (minimal to maximal ranges): FFD<sub>50</sub>/g.
fernalization of the virus with serum antibody may be involved, since IM and SC immunizations also yielded the serum antibody against rabies virus. From the infected terminals, the virus travels along the axon to the perikaryon where it replicates and then infects transsynaptically neural elements having synaptic contact with the infected perikaryon. From the newly infected terminal, the infection progresses to second, and in a similar way, to third and fourth order neurons. CSF drains mostly into the blood, deep cervical lymph nodes and lymphatics located in the connective tissue sheaths of the peripheral nervous system [6, 7], and partly drains into fluid spaces lying along the spinal nerve roots and peripheral nerves [26]. CSF is in direct contact with the extracellular fluid of the CNS including synapses, so the analysis of CSF has been used to study synaptic function and pathology [8, 9]. Furthermore, several compounds injected into the spinal subarachnoid space can be found within the perineurial sheaths and endoneurium of peripheral nerves after having entered dorsal and ventral root ganglia [26]. These findings suggest that a centrifugal spread of CSF containing specific antibodies takes place into extracellular spaces of the neurons of the CNS, DRG, and peripheral nerve fibers, thus the cell to cell spread of rabies virus within the nervous tissues was interrupted by IC immunization. In conclusion, the present experiment suggests that intrathecal immunization is more effective than subcutaneous or intramuscular immunization as pre- and post-exposure vaccination against rabies of animals and humans.

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