

## Comparison of Antibody Titers in Rabbits Following Immunization with Inactivated Influenza Virus via Subarachnoidal or Subcutaneous Route

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**ABSTRACT.** Rabbits were immunized with inactivated influenza virus via the subarachnoidal (SA) or subcutaneous (SC) route, and the antibody titers in cerebrospinal fluid (CSF) and serum were assayed. There were no nervous signs or morphological lesions related to SA immunization. In the SC group, the antibody titer was elevated in serum, but not elevated in CSF. In the SA group, the antibody titer was significantly elevated in serum and even in CSF, and their antibody titers were much greater than in the SC group. The present results suggest that intrathecal immunization is more effective than SC immunization at inducing a protective immune response against the transneural spread of viruses.

**KEY WORDS:** cerebrospinal fluid, influenza virus, intrathecal immunization.

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Many neurotropic infectious agents including rabies virus [6], herpes viruses [15], corona viruses [17], Bornavirus [8], and enterovirus [9] invade the central nervous system (CNS) via peripheral nerves (transneural spread). Intranasally inoculated influenza virus (INFV) strains invaded the pons of mice via peripheral nerves [13, 14, 16, 21]. Also, Yao *et al.* [25] reported that encephalitis was observed within 3–5 days after the intranasal infection of new mice with A/Aichi/2/68 INFV (H3N2) strain. The CNS and peripheral nerves are protected from impact by cerebrospinal fluid (CSF) [18]. The CNS is an immunologically privileged site and serum antibody does not influx into CSF under normal conditions due to blood-brain barrier (BBB) [7, 12]; therefore, those neurotropic infectious agents may not meet the antibody response after they invade the nervous tissue. Serum antibody is effective after the pathogens are elicited on inflammation, which increases BBB permeability.

Direct inoculation of antigens into CSF caused intrathecal immunization. We have previously shown that intrathecal immunization with inactivated pseudorabies virus, a neurotropic virus, completely protected the animals against lethal virus challenge, whereas all non-immunized and several subcutaneously immunized mice died after developing neurological signs [20]. Previous reports have shown that immunization via the brain or CSF elicits systemic humoral immune responses in the cervical lymph nodes, spleen, serum and CSF [7, 8, 19]. Furthermore, antigens are more immunogenic when administered into CNS than into conventional extracerebral sites. When CSF and serum antibody responses to albumin, which was administered into CSF or muscle, were compared with respect to the antibody

titer in a rat model with normal BBB function, the CSF/serum titer ratio and the ratio of immunoglobulin G subclasses were both elevated more prominently following CSF administration than intramuscular inoculation [1, 2, 7]. In this study, we compared the magnitude of the antibody responses in serum and CSF to inactivated INFV following immunization via the subarachnoidal (SA) or subcutaneous (SC) route, and examined the pathological effects of SA inoculation of INFV antigens in the brain.

INFV, A/Aichi/2/68 INFV (H3N2) strain, was propagated in allantoic fluid of 10 day-old embryonated chicken eggs at 35°C for 48 hr. Virus was concentrated and purified by high-speed centrifugation, passed through a 10–50% sucrose density gradient, and resuspended in phosphate-buffered saline (PBS). For formalin-inactivated virus vaccines, the purified viruses were treated with 0.1% formalin at 4°C for a week. Protein concentrations of each vaccine were measured and standardized based on optical density (OD) at 280 nm with Protein Assay Kit (Bio-Rad, UK), and hemagglutinin (HA) units as described previously [22]. Each virus contained 100–200 HA units in 100 µg of purified viral proteins. Inactivation of the virus in each vaccine preparation was confirmed by the absence of detectable HA activity following inoculation of the treated materials into 10 embryonated eggs.

Eleven-week-old female New Zealand White rabbits (N=12) were used in this study. Rabbits were divided into 2 groups by the immunization route. Six rabbits were immunized subcutaneously with 250 µg of H3N2 antigen diluted in 0.5 ml of PBS and emulsified with an equal volume of Freund's complete adjuvant. The rabbits were immunized 2 more times at 3-week intervals with the same concentration of H3N2 antigen with Freund's incomplete adjuvant as a boost. The other six rabbits were immunized by SA injection of 250 µg of antigen diluted in 0.5 ml of PBS without

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adjuvant under anesthesia with Nembutal (20 mg/rabbit). The rabbits were also immunized 2 more times in the same manner as the first immunization at 3-week intervals. In total, the rabbits in both groups were immunized 3 times every 3 weeks. Blood was collected from an ear vein and CSF was collected between lumbar vertebrae 5 and 6. CSF and blood were centrifuged at 5,000 rpm for 10 min at 4°C, respectively, and the supernatants were stored at -20°C until used for antibody titration. CSF and blood were collected before every immunization after anesthesia and three weeks after the final immunization, and all the rabbits were sacrificed after final collection. Tissue samples were fixed in 20% neutral-buffered formalin, embedded in paraffin wax, sectioned at 4  $\mu$ m thickness, and stained with hematoxylin and eosin for histological observation.

Antibody titers of all CSF and serum samples were measured using the enzyme-linked immunosorbent assay (ELISA) as described previously [11]. Ninety-six-well flat-bottom microtiter plates (Nunc, Beckton-Dickinson, NJ) were coated with 50  $\mu$ l of INFV antigen (2  $\mu$ g/ml), which was treated with disruption buffer (0.05 M Tris-HCl, pH 7.8; 0.5% TritonX-100; 0.6 M KCl), per well for 2 hr at room temperature (RT), washed twice and blocked with 100  $\mu$ l of 1% BSA fraction V (Roche, Germany) in PBS for 1 hr at RT. For the antibody titration, 50  $\mu$ l of diluted serum was added to each well in duplicate and incubated for 1 hr at RT. Serum from non-vaccinated rabbits was used as a negative control. After washing, 50  $\mu$ l of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad, UK), diluted 1:2000 in 0.5% BSA fraction, was added to each well for 1 hr at RT. After washing, 100  $\mu$ l of substrate (55 mM 3,3',5,5'-tetramethylbenzidine, 0.03% H<sub>2</sub>O<sub>2</sub>, and 50 mM citric acid buffer pH 4.0) was added to each well for 15 min at RT. After incubation, the reaction was stopped by adding 50  $\mu$ l of 2N H<sub>2</sub>SO<sub>4</sub> and the OD was read at 405 nm.

No animals died or showed any clinical signs related to the immunization in the SC or SA group. There were no specific lesions in the brain and spinal cord of rabbits of either group immunized with inactivated H3N2 antigen. The antibody titers in the serum and CSF of immunized rab-

bbits are shown in Fig. 1. In the serum, the titer was elevated following SC immunization and boosted by additional immunization. The titers also rose after SA immunization and remained higher in the SA group than the SC group, although no adjuvant was used for SA immunization. In the CSF, antibody titers were remarkably higher for rabbits in the SA group than the SC group. Subcutaneously immunized rabbits showed negligible antibody titers in CSF even after the third immunization. The mean ratio of CSF/serum for antibody after the third immunization increased about 70-fold in SA-immunized rabbits as compared to those immunized subcutaneously. Thus, these results showed that SA immunization induced a much stronger antibody-based response in CSF as well as in serum.

In the present study, we assessed the clinical safety and immunogenicity of intrathecal immunization, and compared the magnitude of CSF and serum antibody titers following SA or SC immunization to rabbits with formalin-inactivated INFV. There were no clinical and histological abnormalities due to SA immunization. After SA immunization, antibody titers in serum increased much faster and greater than those after SC immunization, even though adjuvants were used in SC immunization. Furthermore, the antibody titer in the CSF was also significantly elevated by SA immunization; in contrast, an unremarkable increase of the antibody titer was observed in the CSF of SC immunized rabbits. The antibodies detected in the CSF of a few SC immunized rabbits were considered to derive from a small amount of blood contaminated at CSF collection, since the rabbits which showed antibody titer at the 1st or 2nd collection in CSF did not show any antibody titer in the 2nd and/or 3rd CSF collections (data not shown).

Previous studies have shown that intrathecal inoculation yields a greater antibody response in the serum or CSF than extracerebral inoculation for a variety of inert antigens, including bacterial toxin and xenogenic albumin, and strong immunogenicity of CSF-administered antigen has been observed under a wide variety of conditions irrespective of whether the BBB was disrupted or not [7, 10, 15, 19]. We previously reported that higher serum antibody titers were

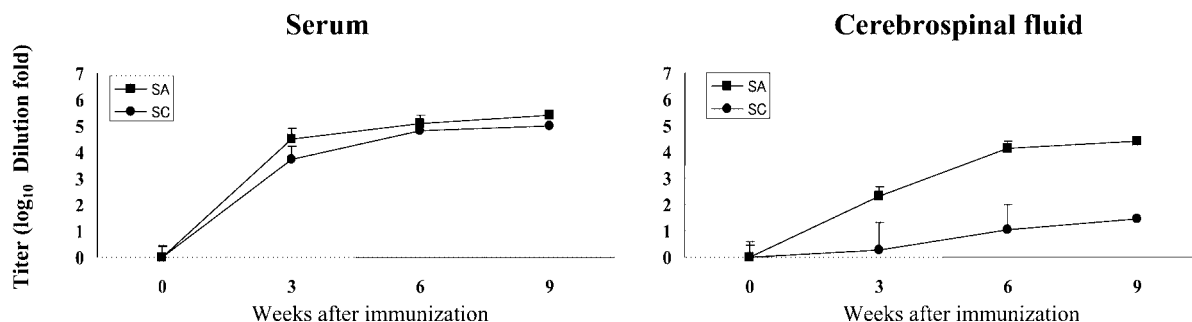


Fig. 1. Antibody titers in the serum and cerebrospinal fluid of immunized rabbits. The rabbits ( $n=6$  per group) were immunized with inactivated H3N2 antigen 3 times at 3-week intervals by subarachnoid (SA) or subcutaneous (SC) route, respectively. The titer in each sample was determined by ELISA, and geometric mean titers were calculated and plotted against time. The antibody titer is expressed as the end-point sample dilution. Each bar represents the geometric means of a group ( $\pm$  standard deviation).

observed in mice after intracerebral immunization with inactivated pseudorabies virus than SC immunization [20]. 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 [1, 2, 7]. CSF drains mostly into the blood, deep cervical lymph nodes and lymphatics located in the connective tissue sheaths of the peripheral nervous system [1, 2], and partly drains into fluid spaces lying along the spinal nerve roots and peripheral nerves [3, 4, 18]. Thus, intracerebrally injected soluble protein antigens are known to drain into the cervical lymph nodes and initiate antigen-specific immune responses [12]. The enhanced immunogenicity of CSF-administered antigen is considered a normal characteristic of brain/immune system interactions, and does not depend on pathological processes such as acute opening of the BBB after infection. In this study, elevated CSF antibody titers in the CNS immunized rabbits strongly indicated that the enhanced response in CSF should depend on events initiated within the CNS, suggesting that intrathecal synthesis as the most likely source of immunoglobulin, because antibody was rarely detected in subcutaneously immunized rabbits. Intrathecal antibody production has been reported in the normal brain [1] as well as in brains with increased BBB permeability or other pathologies of the CNS [5, 23, 24], and these findings appear due to intrathecal antibody synthesis initiated within the CNS, since antigens injected into the blood or nasal submucosa failed to elicit a similar response [1, 2]. Further studies are needed to analyze the immunoglobulin subclass of the antibody raised in CSF and to specify the cell population contributing to intrathecal antibody synthesis.

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## REFERENCES

1. Cserr, H. F., DePasquale, M., Harling-Berg, C. J., Park, J. T. and Knopf, P. M. 1992. *J. Neuroimmunol.* **41**: 195–202.
2. Cserr, H. F. and Knopf, P. M. 1992. *Immunol. Today* **13**: 507–512.
3. Davidsson, P., Jahn, R., Bergquist, J., Ekman, R. and Blennow, K. 1996. *Mol. Chem. Neuropathol.* **27**: 195–210.
4. Davidsson, P., Puchades, M. and K. Blennow. 1999. *Electrophoresis* **20**: 431–437.
5. Doherty, P. C., Allan, J. E., Lynch, F. and Ceredig, R. 1990. *Immunol. Today* **11**: 55–59.
6. Finke, S. and Conzelmann, K. K. 2005. *Virus Res.* **111**: 120–131.
7. Gordon, L. B., Knopf, P. M. and Cserr, H. F. 1992. *J. Neuroimmunol.* **40**: 81–88.
8. Gosztanyi, G. and Ludwig, H. 1995. *Curr. Top. Microbiol. Immunol.* **190**: 39–73.
9. Ha-Lee, Y. M., Dillon, K., Kosaras, B., Sidman, R., Revell, P., Fujinami, R. and Chow, M. 1995. *J. Virol.* **69**: 7354–7361.
10. Harling-Berg, C., Knopf, P. M., Merriam, J. and Cserr, H. F. 1989. *J. Neuroimmunol.* **25**: 185–193.
11. Kida, H., Brown, L. E. and Webster, R. G. 1982. *Virology* **122**: 38–47.
12. Ling, C., Sandor, M. and Fabry, Z. 2003. *J. Neuroimmunol.* **141**: 90–98.
13. Matsuda, K., Shibata, T., Sakoda, Y., Kida, H., Kimura, T., Ochiai, K. and Umemura, T. 2005. *J. Gen. Virol.* **86**: 1131–1139.
14. Matsuda, K., Park, C. H., Sunden, Y., Kimura, T., Ochiai, K., Kida, H. and Umemura, T. 2004. *Vet. Pathol.* **41**: 101–107.
15. Panda, J. N., Dale, H. E., Loan, R. W. and Davis, L. E. 1965. *J. Immunol.* **94**: 760–764.
16. Park, C. H., Ishinaka, M., Takada, A., Kida, H., Kimura, T., Ochiai, K. and Umemura, T. 2002. *Arch. Virol.* **147**: 1425–1436.
17. Perlman, S. 1998. *Adv. Exp. Med. Biol.* **440**: 503–513.
18. Pettersson, C. A. V. 1993. *Acta Neuropathol.* **86**: 636–644.
19. Santos, T. Q. and Valdimarsson, H. 1982. *J. Neuroimmunol.* **2**: 215–222.
20. Shin, J. H., Sakoda, Y., Kim, J. H., Tanaka, T., Kida, H., Kimura, T., Ochiai, K. and Umemura, T. 2006. *Microbiol. Immunol.* **50**: 823–830.
21. Shinya, K., Shimada, A., Ito, T., Otsuki, K., Morita, T., Tanaka, H., Takada, A., Kida, H. and Umemura, T. 2000. *Arch. Virol.* **145**: 187–195.
22. Takada, A., Matsushita, S., Ninomiya, A., Kawaoka, Y. and Kida, H. 2003. *Vaccine* **21**: 3212–3218.
23. Tourtellotte, W. 1970. *J. Neurol. Sci.* **10**: 279–304.
24. Tyor, W. R., Moench, T. R. and Griffin, D. E. 1989. *J. Neuroimmunol.* **24**: 207–215.
25. Yao, D., Kuwajima, M. and Kido, H. 2003. *J. Med. Invest.* **50**: 1–8.