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Experimental intracerebral vaccination protects mouse from a neurotropic virus by attracting antibody secreting cells to the CNS

Hyunkyoung Lee, Yuji Sunden*, Kenji Ochiai, Takashi Umemura

Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, N18 W9, Sapporo 060–0818, Hokkaido, Japan

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ABSTRACT

In previous studies, we showed that intracerebrally (IC) immunized mice had antigen-specific antibodies (Abs) in cerebrospinal fluid and could survive lethal doses of transneurally spreading viruses. To better understand the mechanisms behind this, immune responses in both the central nervous system (CNS) and lymphoid organs following intracerebral immunization against pseudorabies virus (PRV) were investigated by focusing on antibody secreting cells (ASCs). IC immunized mice had significantly higher PRV-specific serum Abs and neutralizing Abs titers than SC immunized mice. Spleen and cervical lymph nodes (CLNs) of IC immunized mice produced significantly more PRV-specific Abs than that of SC immunized mice. ASCs, immunoglobulin and mRNAs of IgG, CXCL9, 10, 13 and BAFF were predominantly detected in the brain of IC immunized mice, but not in SC immunized mice. IC immunized mice (86%) survived more than subcutaneously (SC) immunized mice (33%) by suppression of virus propagation, when PRV was inoculated directly into the brain. In conclusion, IC immunization induced more effective immune responses to protect the CNS from PRV infection by attracting ASCs into the CNS and inducing much more PRV-specific serum neutralizing Abs. This approach may have important implications as a novel treatment procedure for neurotropic virus infections in both humans and animals.

1. Introduction

The central nervous system (CNS) is considered to have immune privilege. The approach of immune cells or circulating proteins to the CNS is interrupted due to the presence of the blood–brain barrier (BBB) and the absence of classically defined lymphatics. In addition, the CNS immune response is restricted by the relative deficiency in expression of major histocompatibility complex (MHC) molecules. However, there have been many clues that the CNS can communicate with peripheral immune system by afferent and efferent arms. Antigens (Ags) within the CNS are transported to draining lymph nodes and spleen, and Ag-specific immune cells can reach both the inflamed and normal CNS [1–5].

Immunization against transneurally spreading viruses via an extracerebral route could have a limited protective effect, as it may be restricted in virus neutralization before the viruses invade the nervous system. Thus, to prevent the transneural spread of viruses, it is important to induce an effective immune response within the nervous system. In this study, intracerebral (IC) vaccination was used to induce CNS immune response, and both the CNS and peripheral immune responses following IC vaccination against pseudorabies virus (PRV) were investigated. PRV, belonging to alphaherpesvirus, is a neurotropic virus that causes Aujeszky's disease in domestic pigs, resulting in economic losses worldwide. Research regarding PRV has been mainly focused on herpesvirus biology and the tracing of neuronal pathways, as well as the control of Aujeszky's disease [6]. PRV causes cytopathic changes in infected brainstem neurons in rodents [7]. Therefore, antibodies (Abs) are thought to be critical for PRV control, because infections with cytopathic viruses are mainly controlled by soluble mediators, such as Abs and interferons [8,9].

The importance of B cells or Abs has been emphasized in research using B cell knock-out mouse, or the adaptive transfer of B cells or Abs in infections and vaccinations of various neurotropic viruses [10–13]. The contribution of B cells within the CNS on the prevention of persistence or recurrence of CNS virus is well documented [5,11,14–17]. Maintenance of Abs over long time periods requires the persistent presence of cells secreting them. These cells are antibody secreting cells (ASCs) known as plasma cells [18–20].

As delivery of peripheral Abs or drugs to the brain is hindered by the restrictive BBB, IC infusion has been tried to improve the approach of drugs or Abs into the brain for the treatment or prevention of brain disease [21,22]. Ovalbumin was more immunogenic when infused into the brain or cerebrospinal fluid (CSF) than when infused into extracerebral sites [23]. Ag introduction into the CNS resulted in intrathecal Abs synthesis by the B lymphocytes lineage

* Corresponding author at: Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, N18 W9, Kita18, Nishi 9, Sapporo 060–0818, Hokkaido, Japan. Tel.: +81 11 706 5193; fax: +81 11 706 5194.
E-mail address: sunden@vetmed.hokudai.ac.jp (Y. Sunden).

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[24]. From these reports, we thought that Ag introduced into brain would induce effective peripheral and CNS immune responses.

Previously, we demonstrated that intracerebrally (IC) introduced inactivated PRV or rabies virus (RV) induced more effective protection against intramuscularly inoculated PRV or RV than subcutaneous (SC) immunization in mice. The higher virus-specific serum Abs of IC immunized mice were thought to contribute to the efficient protection. The findings that PRV and RV were not detected in the nervous system of IC immunized mice suggested that the viruses were blocked before invading the nervous system from a peripheral system [25,26]. We therefore questioned whether IC immunization could induce a protective immune response within the CNS, where PRV replicates and where the response is most important. To clarify the contribution of IC introduced inactivated PRV, we analyzed the immune responses in the brain and periphery after IC or SC immunization, while focusing on the role of ASCs.

2. Materials and methods

2.1. Virus and mice

Yamagata-S81 strain of PRV was propagated using CPK cell, a cell line of swine kidney origin. The cells were incubated at 37 °C in Dulbecco's Modified Eagle's Media (Nissui, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 μg/ml streptomycin, and 50 IU/ml penicillin. The propagated virus were purified by ultracentrifuge in sucrose gradient, and inactivated by formalin as previously described [25]. Female BALB/c mice of six weeks of age (Nippon Clea Inc., Japan) were maintained in a specific pathogen-free environment and immunized intracerebrally or subcutaneously twice over a two-week interval. Mice were injected with 100 μg of inactivated PRV (30 μl in volume for the intracerebral route, 100 μl in volume for the subcutaneous route). The intracerebral injection was performed with the syringe, which was provided with a penetrating depth controller. The injection site was restricted to the right frontal lobe at 3 mm depth. At two weeks after second immunization, mice were challenged by intracerebral inoculation of 1×10^2 PFU PRV and monitored daily. Survival rates were assessed for three weeks after virus challenge. Virus challenge experiment was done twice with total 15 mice per group. The necropsy was performed at 7, 14 days after first immunization, 5, 10, 14, 21 days after second immunization and 60 h after virus challenge. After blood collection, mice were perfused systemically with PBS, and then cervical lymph nodes (CLNs), spleen and brain were collected. All animal experiment procedures followed the guidelines of the Institutional Animal Care and Use Committee, Hokkaido University, and the Association for Assessment and Accreditation of Laboratory Animal Care International Standards.

2.2. Analysis of serum antibodies

PRV-specific serum antibodies (Abs) were evaluated by ELISA and neutralizing test with the sera from 3–5 mice per time point from 2–3 experiments. The ELISA was performed as previously described [27]. The 96-well ELISA plates were coated for 2 h at RT with 2×10^6 PFU/well of PRV diluted in disruption buffer (0.05 M Tris–HCl, pH 7.8; 0.5% TritonX-100; 0.6 M KCl). The plates were blocked with PBS containing 10 mg/ml BSA for 1 h at RT, and washed twice with 0.05% Tween 20 PBS (PBS-T). After washing, sera diluted in 0.05% Tween 20 PBS containing 5 mg/ml BSA (BSA_5T) were added and incubated for 1 h at RT. HRP-conjugated anti-mouse Ig (Biosource, USA), IgG1 or IgG2a (Santa cruz biotechnology, USA) diluted in BSA_5T by 1:2000 was added after being washed four times and incubated for 1 h at RT. After being washed four times, the substrate solution TMB (Pierce, USA) was incubated for 30 min at RT and the reaction was stopped by 2 M H_2SO_4. The absorbance was read at 450 nm. The neutralizing test was performed as previously described [28]. For complement inactivation, sera were incubated for 30 min at 56 °C and stored at −20 °C. Sera were diluted two-fold in 96-well cell culture plates, mixed with 2.8×10^2 PFU PRV in a 100 μl volume and incubated for 24 h at 37 °C in humidified atmosphere containing 5% CO_2. After incubation, 100 μl of cell suspension containing 1×10^4 cells was added and plates were incubated for 5 days at 37 °C in 5% CO_2. Thereafter, cells were monitored for the PRV-specific cytopathic effect (CPE) and neutralization titers were calculated as the $\log_2$ of the reciprocal of the highest dilution resulting in complete neutralization.

2.3. Lymphocyte isolation

Lymphocytes were isolated from CLNs and spleen of 3–5 mice per time point from 2–3 experiments. To isolate leukocytes from the organs, tissues were dissected in Hank's buffered saline solution (HBSS), and then pressed through a nylon mesh (BD biosciences, USA). Cell suspensions were overlaid on 60% Percoll (GE healthcare Bio-Science Corp., USA) and then centrifuged at 2000 rpm for 20 min. The lymphocytes harvested from the interface were washed with HBSS twice, and were counted after staining with 0.1% trypan blue.

2.4. Analysis with lymphocyte

2.4.1. ELISA with lymphocytes culture supernatants

PRV-specific Abs secretion from each lymphoid organ was compared as described previously [29]. The lymphocytes isolated were cultured in RPMI 1640 media (Gibco, USA) supplemented with Glutamax, 20% FBS, 50 μg/ml streptomycin, and 50 IU/ml penicillin. 6×10^6 cells/300 μl was suspended in 24-well cell culture plates in duplicate. After incubation for 3 days, cell culture supernatants were collected and PRV-specific Abs were analyzed with ELISA.

2.4.2. ELIspot

The frequency of PRV-specific IgG antibody secreting cells from spleen was measured with an enzyme-linked immunospot assay (ELIspot) in nitrocellulose-bottomed 96-well plates (MultiScreen-HTS, Millipore, USA). Each well was pre-wetted with 15 μl of 35% ethanol (v/v in Milli-Q) for 1 min. The plates were washed with 150 μl sterile PBS three times before ethanol evaporation. Each well was coated with inactivated PRV (20 μg/ml in PBS, 100 μl/well). After incubation overnight at 4 °C, plates were washed once with 10% FBS-RPMI 1640 media (200 μl/well) and blocked with 10% FBS-RPMI 1640 media (200 μl/well) for 2 h at 37 °C. Lymphocytes, adjusted to 5×10^6/ml and serially diluted 1:2 in three steps in culture medium, were then seeded out in duplicate wells (200 μl/well) and incubated for 18 h at 37 °C in a humidified atmosphere containing 5% CO_2. The plates were then washed twice with PBS and three times with PBS-T. Then, 100 μl of Biotin-SP-conjugated affinipure rabbit anti-mouse IgG (H + L) (1: 5000 in 10% FBS-PBS, Jackson Immunoresearch, USA) was added to each well and incubated overnight at 4 °C. Thereafter, the plates were washed four times with PBS-T, and 100 μl of HRP-conjugated biotin–avidin complex solution (diluted in 10% FBS-PBS in accordance with the manufacturer's instructions, StreptABComplex/HRP, BD, USA) was added and incubated in the dark for 1 h at RT. The plate was washed four times with PBS-T and twice with PBS. To reveal the peroxidase staining, AEC substrate was incubated for 15 min. When spots appeared, the wells were thoroughly rinsed under tap water and air-dried overnight. The spots, each one corresponding to one antibody-secreting cell, were counted under a stereomicroscope (SZ4045TR; Olympus, Tokyo, Japan).