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Protective Immunity Induced by Systemic and Mucosal Delivery of DNA Vaccine Expressing Glycoprotein B of Pseudorabies Virus

Yoon, Hyun A, Young Woo Han, Abi George Aleyas, Junu Abi George, Seon Ju Kim, Hye Kyung Kim, Hee Jong Song, Jeong Gon Cho, and Seong Kug Eo*

Department of Microbiology, College of Veterinary Medicine and Bio-Safety Research Institute, Chonbuk National University, Jeonju 561-756, Korea

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A murine model immunized by systemic and mucosal delivery of plasmid DNA vaccine expressing glycoprotein B (pCIgB) of pseudorabies virus (PrV) was used to evaluate both the nature of the induced immunity and protection against a virulent virus. With regard to systemic delivery, the intramuscular (i.m.) immunization with pCIgB induced strong PrV-specific IgG responses in serum but was inefficient in generating a mucosal IgA response. Mucosal delivery through intranasal (i.n.) immunization of pCIgB induced both systemic and mucosal immunity at the distal mucosal site. However, the levels of systemic immunity induced by i.n. immunization were less than those induced by i.m. immunization. Moreover, i.n. genetic transfer of pCIgB appeared to induce Th2-biased immunity compared with systemic delivery, as judged by the ratio of PrV-specific IgG isotypes and Th1- and Th2-type cytokines produced by stimulated T cells. Moreover, the immunity induced by i.n. immunization did not provide effective protection against i.n. challenge of a virulent PrV strain, whereas i.m. immunization produced resistance to viral infection. Therefore, although i.n. immunization was a useful route for inducing mucosal immunity at the virus entry site, i.n. immunization did not provide effective protection against the lethal infection of PrV.

Keywords: Pseudorabies virus, DNA vaccine, systemic delivery, mucosal delivery, protective immunity

The pseudorabies virus (PrV) is a porcine alphaherpesvirus that causes a fatal disease known as Aujeszky's disease (AD) in swine. AD is one of the most significant infectious diseases in the swine industry [20]. Attempts have been made to control the outbreak of AD in swine and to reduce the associated economic losses in the swine industry using active immunization with modified live or inactivated vaccine [18, 27]. Attenuated vaccines, which generally induce long-lasting immunity, carry a risk of insufficient attenuation and/or genetic instability. Moreover, inactivated vaccines are also less efficient and require repeat doses. In more recent years, vaccination with plasmid DNA that encodes a foreign antigen has been one of the most significant advances in immunology and vaccine research. Similar successes have been reported in the use of plasmid DNA for vaccination of pigs to provide protective immunity against virulent PrV infection [5, 6, 9, 13, 22, 37, 39]. Generally, a combination of plasmid DNA expressing three major glycoproteins (gB, gC, and gD) has been investigated in PrV DNA vaccination [13, 37]. These glycoproteins are involved in the essential steps of viral infection [20]. Several B-cell epitopes detected on PrV gB and gC glycoproteins [29, 30, 42, 43] and a T-cell epitope detected on PrV gC induce both humoral and cytotoxic responses [29, 30]. PrV gD induces production of strongly neutralizing antibodies and weak cytotoxic responses [36].

A better understanding of the immunological parameters relevant to protection is a prerequisite for improving the effectiveness of antiherpes vaccines including PrV vaccine. Cell-mediated immunity biased to Th1-type is believed to be an important protective effector mechanism against PrV infection [2, 8, 35]. Thus, vaccination to induce Th1-biased immunity has been reported to provide effective protection against virulent PrV infection [40]. Among the three major glycoproteins, plasmid DNA expressing gB protein injected intramuscularly (i.m.) into animals induced Th1-biased immunity, which subsequently provided the most effective protection against a virulent virus challenge [39]. Moreover, incorporation of immunomodulatory cytokines driving Th1-biased immunity helped enhance resistance against a virulent PrV infection [38, 40]. Furthermore, specific virus-neutralizing serum antibodies are believed to play a substantial role in controlling PrV infection [17, 26]. However, the contribution of mucosal IgA responses

*Corresponding author
Phone: 82-63-270-3882; Fax: 82-63-270-3780;
E-mail: vetvirus@chonbuk.ac.kr

appearing in mucosal tissues, which can be a major entry point for PrV, has not been investigated to date.

The large majority of DNA vaccine studies employ systemic immunization and fail to record if responses also occur at mucosal sites [4]. In animal models, DNA vaccines targeted to the specialized inductive sites of the mucosal immune system have been shown to elicit both systemic and mucosal antibody responses [21], as well as proliferative CTL responses [28]. The nasal mucosa is an important branch of the mucosal immune system since it is generally the first entry site of inhaled pathogens including PrV [3]. Therefore, the nasal mucosa presents an attractive, noninvasive route for DNA vaccine delivery. In the present report, we compared the effectiveness of intranasal (i.n.) PrV DNA vaccine administration with that of intramuscular (i.m.) administration in the induction of Th1/Th2-type immune responses and their ability to provide protective immunity against PrV infection.

Materials and Methods

Animals

Female C57BL/6 (H-2^b) mice, 5 to 6 weeks of age, were purchased from KOATECH (Pyeongtaek, Korea). The mice were maintained in the animal facility at Chonbuk National University under standard conditions according to the Institutional Guidelines. All experiments were performed according to the guidelines of the Committee on the Care of Laboratory Animals Resources, Commission on Life Science, National Research Council.

Viruses and Cells

The pseudorabies virus (PrV) Yangsan (YS) strain, which was a kind gift from the National Veterinary Research and Quarantine Service in Korea, was propagated in a porcine kidney cell line (PK-15) using DMEM supplemented with 2.5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml). The PK-15 cultures were infected with the PrV at a multiplicity of infection (MOI) of 0.01, and incubated in a humidified CO_2 incubator for 1 h at 37°C. After absorption, the inoculum was removed, and 10 ml of a maintenance medium containing 2% FBS was added. Approximately 48–72 h post-infection, cultures of the host cell showing an 80–90% cytopathic effect were harvested. The virus stocks were concentrated by centrifugation at 50,000 ×*g*, titrated by a plaque assay, and stored in aliquots at −80°C until needed.

Plasmid DNA Preparation

Plasmid DNA encoding gB of PrV under the cytomegalovirus (CMV) promoter (pCIgB) has been described in detail elsewhere [38–40]. Plasmid DNA for immunization was purified by polyethylene glycol precipitation as described previously [38–40]. Briefly, cellular proteins were precipitated with one volume of 7.5 M ammonium acetate, followed by isopropanol precipitation of the supernatant. After polyethylene glycol precipitation, the plasmid was extracted three times with phenol-chloroform and precipitated with pure ethanol. The DNA quality was checked by electrophoresis on a 1% agarose gel. The plasmid DNA concentration was measured using a GeneQuant RNA/DNA calculator (Biochrom, Cambridge, U.K.). The amount of endotoxin was determined by the *Limulus amebocyte* lysate (LAL) test (<0.05 EU/μg). The *in vivo* effect of endotoxin and CpG was always addressed by parallel administration of the control vector, pCI-neo (Promega, Madison, WI, U.S.A.).

DNA Vaccination Protocol and Sample Collection

Groups of 5- to 6-week-old female mice were immunized with a PBS (pH 7.2) formulation of the plasmid DNA vaccine encoding the glycoprotein gB *via* either the intranasal (i.n) or intramuscular (i.m) route. For i.m. administration, 100 μg of pCIgB DNA was injected into the anterior tibialis muscle three times at weekly intervals (days 0, 7, and 14). The i.n. immunization was performed three times at weekly intervals (days 0, 7, and 14) by depositing 100 μg of pCIgB dissolved in a total volume of 20 μl of PBS (pH 7.2) onto the nares of deeply anesthetized mice. Control mice were given empty vector plasmid DNA, pCI-neo, dissolved in PBS. Serum samples were collected on the seventh day after each immunization by retro-orbital bleeding and stored at −80°C until needed. Vaginal lavage fluid samples were obtained by introducing 100 μl of PBS (pH 7.2) into the vaginal canal and recovering it with a micropipette. Vaginal lavages were collected once a day for 3 days on day 7 post-immunization and combined.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was employed to compare the expression levels of glycoprotein gB following either i.m. or i.n. administration of pCIgB using the Bio-Rad Laboratories MiniOpticon real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.) [15, 16, 24, 33]. The total RNA was extracted from tissues (anterior tibialis muscle, spleen, and popliteal lymph node for i.m. injection, and lung, spleen, mediastinal, and cervical lymph nodes for i.n. administration) using the QuickGene RNA tissue kit (Fuji Film, Tokyo, Japan), according to the manufacturer's instructions. The contaminating plasmid DNA was removed by treatment with RQ1-RNase-free DNase (Sigma, St. Louis, MO, U.S.A.). Following reverse-transcription of 500 ng of total RNA, the resulting cDNAs were used for real-time PCR amplification. For detecting the gB gene, the forward primer, 5'-ACGGCACGGGCGTGATC-3', and the reverse primer, 5'-ACTCGCGGTCCTCCAGCA-3', were used [41]. PCR amplification was performed with a DyNAmo SYBR Green 2-Step qRT-PCR kit (Fynnzymes, Espoo, Finland) by using initial denaturation (95°C, 15 min) and then 40 cycles of denaturation (94°C, 15 sec) and annealing (60°C, 30 sec) for all mRNAs. A standard curve was generated by plotting threshold cycle values against serially diluted plasmid DNA encoding gB. The copy number of the experimental samples was determined by interpolating threshold cycles values into the standard curve. All data were analyzed using the MJOPTICON MONITOR version 3.1 analysis software.

ELISA for PrV-specific Antibody, IgG, IgG1, IgG2a, and IgA

A standard enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of PrV-specific antibodies in the serum and vaginal lavage [total immunoglobulin G (IgG), IgG1, IgG2a, and vaginal IgA]. Briefly, ELISA plates were coated overnight at 4°C with an optimal dilution (0.5–1.0 μg/well) of semi-purified PrV antigen in the sample wells, and goat anti-mouse IgG/IgG1/IgG2a

(Southern Biotechnology Associate Inc., Birmingham, AL, U.S.A.) or rabbit anti-mouse IgA (Zymed, San Francisco, CA, U.S.A.) in the standard wells. The viral antigen for coating was prepared by semi-purification of the viral stock by centrifugation at 50,000 ×*g* after treatment with 0.5% Triton X-100 (Sigma, St. Louis, MO, U.S.A.) [2]. The plates were washed three times with PBS-Tween 20 (PBST) and blocked with 3% dehydrated milk. The samples were serially diluted twofold, incubated for 2 h at 37°C, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG/IgG1/IgG2a for 1 h. To measure the IgA level in the vaginal lavage fluid samples, biotinylated goat anti-mouse IgA was added for 2 h at 37°C, followed by the addition of peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). The color was developed by the addition of a suitable substrate (11 mg of 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid in a mixture of 25 ml of 0.1 M citric acid, 25 ml of 0.1 M sodium phosphate, and 10 µl of hydrogen peroxide). The concentration of PrV-specific antibodies was determined using an automated ELISA reader and the SOFTmax Pro4.3 program (Spectra MAX340, Molecular Device, Sunnyvale, CA, U.S.A.).

Th1- and Th2-type Cytokine ELISA Following *In Vitro* Stimulation of CD4+ T Cells

Two weeks after the final immunization, mice were sacrificed to prepare splenocytes and popliteal/cervical lymph node (LN) cells. The erythrocytes were depleted by treating the single-cell suspensions with ammonium-chloride-containing Tris buffer (NH_4Cl-Tris) for 5 min at 37°C. These cells of spleen and LNs were used as responder cells. The enriched antigen-presenting cell (APC) populations, which were obtained as described previously [7], were used as stimulators. Briefly, splenocytes from naïve female mice were depleted of erythrocytes, and 10^7 cells in 3 ml were layered over 2 ml of a metrizamide gradient (Accurate Chemical and Sci., Westbury, NY, U.S.A.; analytical grade, 14.5 g added to 100 ml of PBS, pH 7.2). The cells were then centrifuged at 600 ×*g* for 10 min, and the cell interface was collected. The enriched APC population was pulsed with UV-inactivated PrV at 5.0 MOI for 3 h (prior to inactivation). The cells were then washed and counted. The responder cells and the PrV-pulsed APCs were combined at responder-to-stimulator ratios of 5:1, 2.5:1, and 1.25:1 in 200 µl of a RPMI medium. The culture supernatants were harvested after 3 days of incubation. A similar number of responder cells were stimulated with 5 µg of concanavalin A as a polyclonal positive stimulator for 48 h.

ELISA was used to determine cytokine levels in the culture supernatants. The ELISA plates were coated with IL-2, IL-4, and IFN-γ anti-mouse antibodies (Pharmingen, San Diego, CA, U.S.A.; clone no. JES6-1A12, 11B11, and R4-6A2, respectively) and incubated overnight at 4°C. The plates were washed three times with PBST and blocked with 3% nonfat-dried milk for 2 h at 37°C. The culture supernatant and standards for recombinants IL-2, IL-4, and IFN-γ protein (Pharmingen) were added to the plates and incubated overnight at 4°C. Biotinylated IL-2, IL-4, and IFN-γ antibody (Pharmingen; clone no. JES6-5H4, BVD6-24G2, and XMG1.2, respectively) were then added and further incubated for 2 h at 37°C. The plates were then washed and incubated with peroxidase-conjugated streptavidin (Pharmingen) for 1 h, followed by color development with a substrate (ABTS) solution. The concentrations of cytokines were determined using an automated ELISA reader and SOFTmax Pro4.3 by comparison with two concentrations of standard cytokine protein.

Virus Challenge Experiment

Two weeks after the final immunization, the immunized mice were infected i.n. with the virulent PrV YS strain (10 LD_{50}). The challenged mice were examined daily to determine the number of dead animals. The challenged mice generally began to elicit clinical signs 3- to 4-days post-challenge.

Statistical Analysis

Where specified, the data were analyzed for statistical significance using Student's *t*-test. A p value <0.05 was considered significant.

RESULTS

Comparison of the Expression Levels of Glycoprotein gB Following Systemic and Mucosal Delivery

Before evaluating the nature of protective immunity induced by systemic and mucosal delivery of pCIgB, we determined the expression level of the gB gene using qRT-PCR three days after administrating pCIgB *via* i.m. and i.n. routes. The total RNA, which had been extracted from tissues indicated in Fig. 1, was reverse-transcribed. The resulting cDNA was then used for real-time PCR amplification using PrV gB-specific primers. As shown

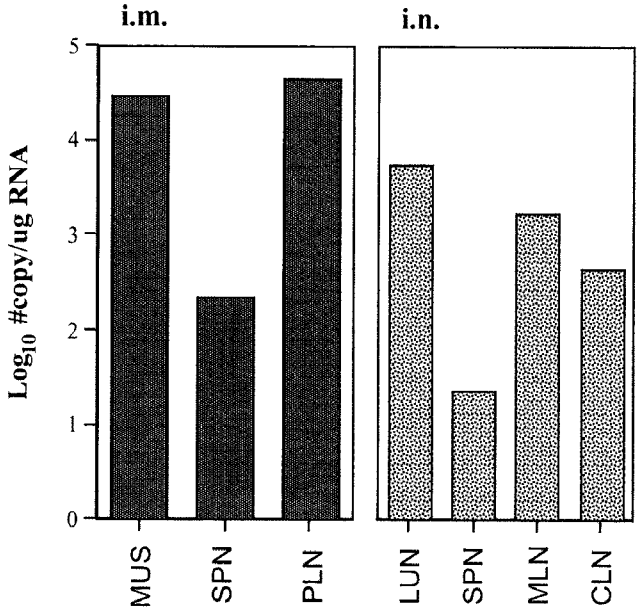

Fig. 1. Quantitative analysis of expression of PrV gB gene in lymphoid and non-lymphoid tissues following systemic and mucosal delivery of pCIgB DNA vaccine.

Three days following i.m. and i.n. administration of pCIgB DNA vaccine, the total RNA was extracted from indicated tissues (MUS: muscle; SPN: spleen; PLN: popliteal lymph node; LUN: lung; MLN: mediastinal lymph node; CLN: cervical lymph node) and employed to quantitative real-time PCR, as described in Materials and Methods. The height of each bar represents the average of copy number of PrVgB gene detected in the indicated tissue from two mice.

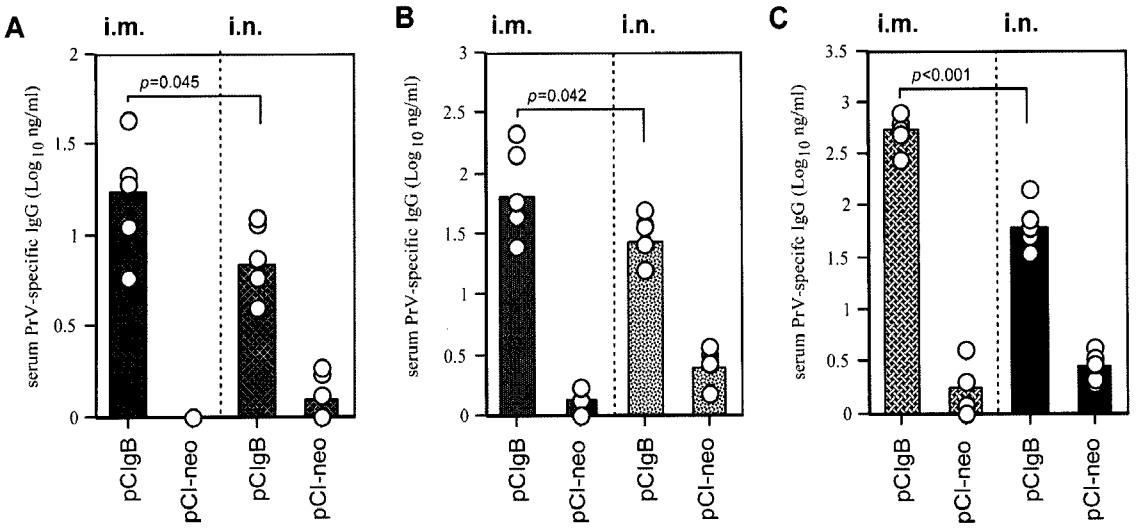

Fig. 2. Serum PrV-specific IgG levels in animals immunized by systemic (i.m.) and mucosal (i.n.) delivery of pCIgB DNA vaccine.
Groups of C57BL/6 (H-2^b) mice were immunized on three occasions at 7-day intervals *via* i.m. and i.n. routes. On the 7th day after each immunization (**A**, **B**, and **C**), PrV-specific IgG levels in sera were determined by conventional ELISA. The circles on the graph represent the individual serum IgG levels, and the height of the bar shows the average for each group. *P*-values in the graphs were statistically calculated by Student's *t*-test.

in Fig. 1, the expression of gB gene following i.n. administration of pCIgB showed marginally reduced levels than that of i.m. injection. However, there was no significant difference between i.m. and i.n. administration. In particular, the deposited site of pCIgB (muscle and lung) and its draining LN (popliteal LN for i.m. injection, mediastinal and cervical LN for i.n. administration) showed a higher copy number of gB gene transcripts than spleen of recipient mice three days after immunization. Therefore, this indicates that plasmid DNA vaccine encoding PrV gB was successfully expressed after the i.m. and i.n. administration.

Humoral Responses Induced by Systemic and Mucosal Delivery of PrV gB-encoding DNA Vaccine

To assess humoral responses induced by PrVgB-encoding plasmid DNA administered *via* either a systemic or a mucosal route, groups of mice were immunized with pCIgB by intramuscular (i.m.) and intranasal (i.n.) routes. For i.m. immunization, pCIgB was injected into the

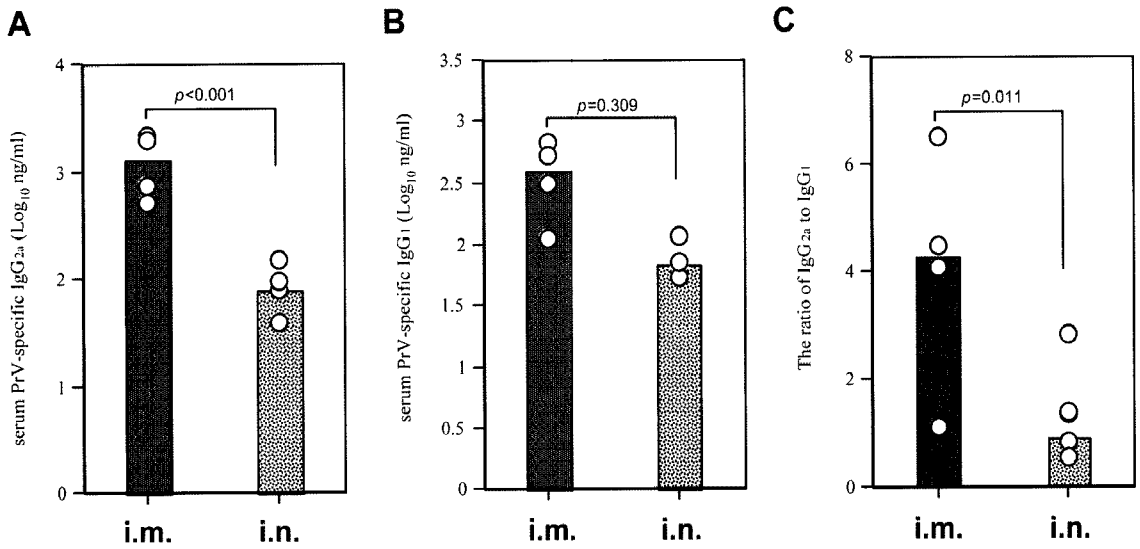

Fig. 3. The levels of serum PrV-specific IgG isotypes (IgG2a and IgG1) in animals immunized by systemic (i.m.) and mucosal (i.n.) delivery of pCIgB DNA vaccine.
Groups of C57BL/6 (H-2^b) mice were immunized on three occasions at 7-day intervals *via* i.m. and i.n. routes. On the 7th day after the final immunization, the levels of PrV-specific IgG isotypes, IgG2a (**A**), IgG1 (**B**), and the ratio of IgG2a to IgG1 (**C**) were determined by conventional ELISA. The circles on the graph represent the individual levels of serum IgG levels, and the height of the bar shows the average for each group. *P*-values in the graphs were statistically calculated by Student's *t*-test.

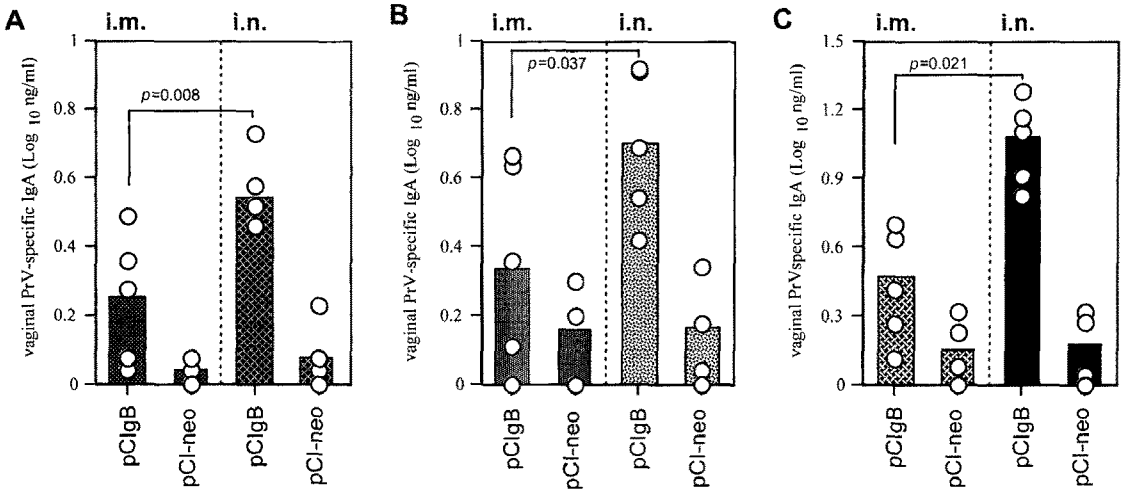

Fig. 4. Vaginal PrV-specific IgA levels in animals immunized by systemic (i.m.) and mucosal (i.n.) delivery of pCIgB DNA vaccine. Groups of C57BL/6 (H-2^b) mice were immunized on three occasions at 7-day intervals *via* i.m. and i.n. routes. On the 7th day after each immunization (**A**, **B**, and **C**), PrV-specific IgA levels in vaginal lavages were determined by conventional ELISA. The circles on the graph represent the individual vaginal IgA levels, and the height of the bar shows the average for each group. *P*-values in the graphs were statistically calculated by Student's *t*-test.

anterior tibialis muscle three times at weekly intervals. The i.n. immunization was performed by depositing pCIgB DNA vaccine onto the nares of deeply anesthetized mice. The PrV-specific IgG levels in sera were then determined on the seventh day after each immunization, as shown in Fig. 2. The control vector, pCI-neo, induced no significant

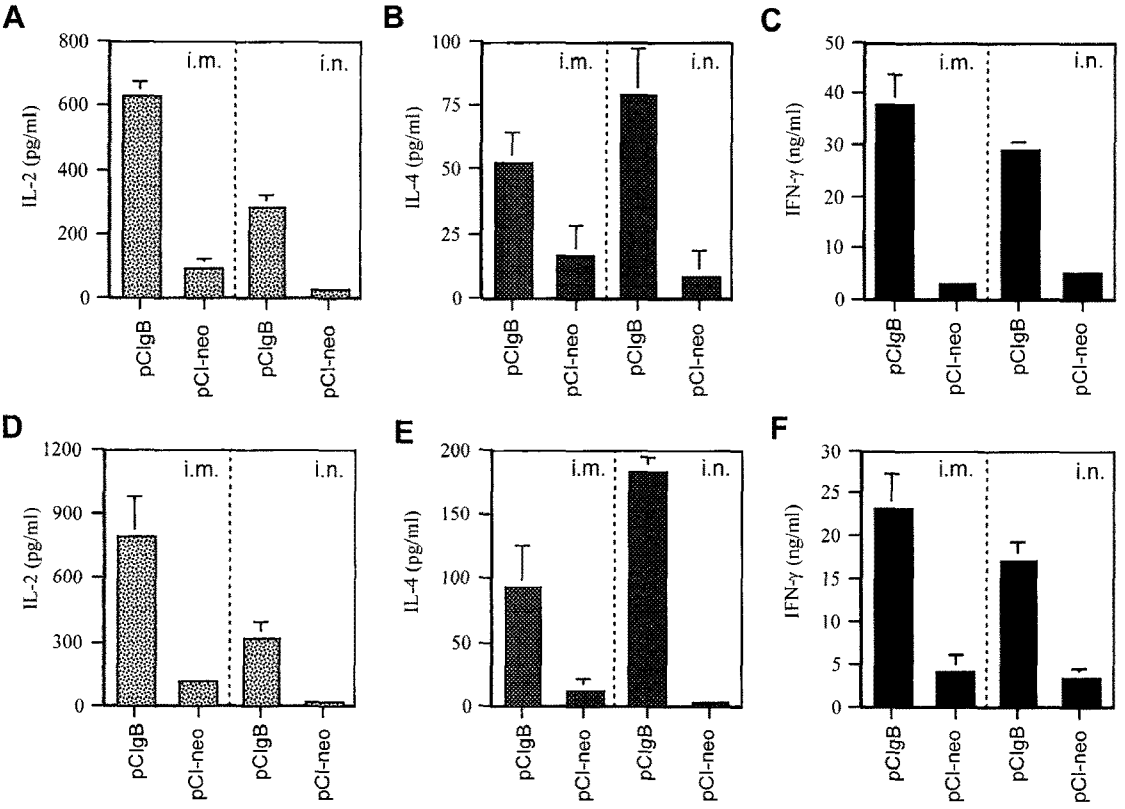

Fig. 5. The profile of Th1- and Th2-type cytokine production (IL-2, IL-4, and IFN-γ) from splenocytes and draining LN (popliteal LN for i.m. and cervical LN for i.n.) cells of animals immunized by systemic (i.m.) and mucosal (i.n.) delivery of pCIgB DNA vaccine. Two weeks after the final immunization, as indicated in Materials and Methods, the responder cells (splenocytes and LN cells from the immunized mice) were mixed with irradiated syngeneic enriched APCs that had been pulsed with UV-inactivated PrV and then incubated for 3 days. Cytokine levels in the supernatants of the stimulated T cells were determined by ELISA. The tests were carried out in quadruplicate wells. The height of each bar represents the average and standard deviation of three independent experiments.

PrV-specific IgG response, whereas pCIgB DNA vaccine administered i.n. or i.m. produced detectable IgG levels after the first immunization (Fig. 2A). Subsequent i.m. and i.n. administration of pCIgB DNA boosted these primary IgG responses (Figs. 2B and 2C). However, i.m. administration of pCIgB DNA vaccine resulted in stronger IgG responses in the sera than i.n. administration. This result indicates that i.n. administration of pCIgB DNA vaccine can induce systemic IgG responses in the sera like i.m. administration, even though less PrV-specific IgG was induced.

Moreover, a different distribution of PrV-specific IgG isotypes, IgG2a and IgG1, was observed following systemic and mucosal delivery of pCIgB DNA vaccine (Fig. 3). Systemic injection of pCIgB elicited higher production of PrV-specific IgG2a isotype than IgG1, and subsequently showed a high level of IgG2a/IgG1 ratio (Th1-type-biased response), whereas i.n. administration showed a lower level of IgG2a/IgG1 ratio by comparable production of both isotypes (Fig. 3C). Therefore, this indicates that systemic injection of DNA vaccine against PrV drives the immunity to be biased to Th1-type, more than mucosal delivery.

In contrast, when IgA responses at mucosal sites were analyzed on the seventh day after each immunization, the levels of induced IgA responses revealed a pattern different from that of serum IgG responses (Fig. 4). None of the mice immunized with the control empty vector had significantly detectable PrV-specific mucosal IgA responses in vaginal lavages. However, the first i.n. administration of pCIgB induced significant secretory IgA responses at the mucosal site that increased with subsequent administration. On the other hand, the i.m. administration of pCIgB induced a much less IgA response. Moreover, the subsequent i.m. administration of pCIgB DNA vaccine did not elicit significant increases. Therefore, these results indicate that both mucosal IgA and serum IgG responses are induced by mucosal genetic transfer of pCIgB DNA vaccine. In contrast, systemic delivery of pCIgB DNA vaccine produced strong serum PrV-specific IgG responses, but not IgA responses at mucosal sites.

Th1- and Th2-type Cytokines Produced from Stimulated CD4+ T Cells

Normally, intramuscular DNA vaccine administration induces Th1-type immune responses against the encoded antigen, which can be judged by the ratio of IgG isotypes and Th1/Th2 cytokine production [11, 14, 23]. Therefore, to further evaluate the nature of the immunity induced by systemic and mucosal delivery of pCIgB DNA vaccine, we determined the Th1 (IL-2 and IFN-γ) and Th2 (IL-4)-type cytokines produced from CD4+ T cells stimulated with the antigen protein, which is known to induce the predominant expansion of immune CD4+ T cells [7]. Following i.m. or i.n. administration of pCIgB, immune T cells obtained from spleen and draining LNs of immunized mice were stimulated with syngeneic APC pulsed with inactivated PrV antigen, and the levels of cytokines in the supernatant of stimulated T cells were then determined, as shown in Fig. 5. There was no significant production of IL-2, IL-4, and IFN-γ in mice administered the control vector. However, Th1- and Th2-type cytokines were significantly produced from CD4+ T cells stimulated with PrV antigen following i.m. and i.n. administration of pCIgB DNA vaccine. Moreover, systemic and mucosal delivery of pCIgB DNA vaccine induced different patterns of Th1- and Th2-type cytokine production. Immunity induced by i.m. administration of pCIgB DNA vaccine was biased toward Th1-type, as determined by production of the Th1-type cytokines, IL-2 and IFN-γ. In contrast, i.n. administration of pCIgB DNA vaccine enhanced production of the Th2-type cytokine IL-4 compared with i.m. administration. Therefore, these results, together with PrV-specific IgG isotypes, indicate that mucosal delivery of pCIgB induces Th2-biased immunity, more so than systemic delivery.

Protective Immunity Against Viral Challenge

To determine whether the different natures of the immunity induced by systemic and mucosal pCIgB DNA vaccine delivery affect the level of protection provided against virulent virus infection, mice given i.m. or i.n. administration of pCIgB DNA vaccine were challenged i.n. with the virulent PrV YS strain (10 LD_{50}) 2 weeks after the final immunization (Fig. 6). All animals immunized i.n. with

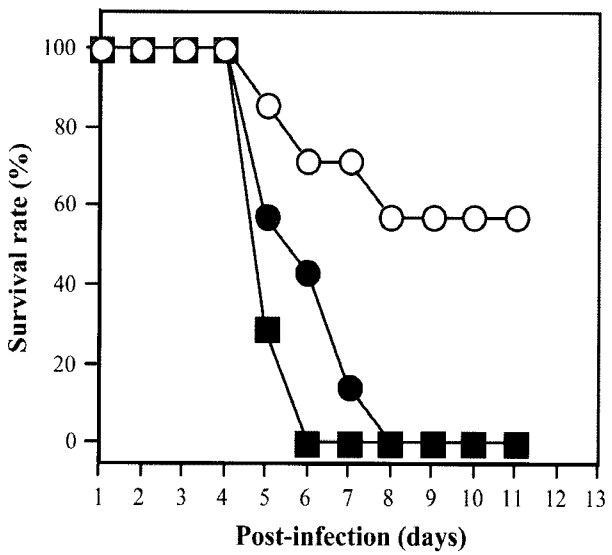

Fig. 6. Susceptibility of animals immunized by systemic (i.m.) and mucosal (i.n.) delivery of pCIgB DNA vaccine to a virulent PrV infection.

Two weeks after the final immunization, groups of mice (n=7) were challenged i.n. with the PrV YS strain (10 LD_{50}). The challenged mice were examined daily to determine the number of dead animals until 11 days post-challenge. ■, pCI-neo control vector (i.m.); ○, pCIgB (i.m.); ●, pCIgB (i.n.).

pCIgB DNA vaccine were sacrificed with a virulent virus infection, despite a delayed death rate, compared with animals given the control vector, pCI-neo. However, i.m. injection of pCIgB DNA vaccine provided significant protection against the viral challenge. Systemic administration of pCIgB DNA vaccine offered protection, with a survival rate of 57.1%. Therefore, systemic delivery of pCIgB DNA vaccine provided more effective protection against virulent virus infection than mucosal delivery.

DISCUSSION

Immunization with naked DNA encoding various genes has proven to be a valuable means of inducing immunity [4]. The great majority of DNA vaccine studies have used the i.m. or gene gun delivery approach to administer the DNA, and this method has produced the best results. Protective immune responses following DNA vaccination have been demonstrated against several diseases and pathogens in both experimental animals and humans using different routes of immunization including i.m., intravenous (i.v.), oral, i.n., and vaginal administration [10, 12, 19]. In the present report, we evaluated the protective immunity induced by systemic and mucosal delivery of DNA vaccine against PrV. We chose i.n. immunization for mucosal delivery of the DNA vaccine. Here, i.n. administration was performed by depositing PrV DNA vaccine onto the nares of deeply anesthetized mice. Systemic delivery by i.m. immunization with the DNA vaccine proved superior to i.n. immunization in the induction of serum PrV-specific IgG levels. However, as noted previously [21], i.m. immunization was less effective than i.n. immunization in the induction of mucosal IgA. In contrast, i.n. immunization induced both systemic and mucosal immunity at a distal mucosal site (vaginal tract). However, the levels of systemic immunity induced by i.n. immunization were less than those induced by i.m. immunization. Moreover, i.n. genetic transfer of PrV DNA vaccine appeared to induce Th2-biased immunity, more so than systemic delivery, as judged by PrV-specific IgG isotypes and Th1/Th2-type cytokines produced by stimulated T cells. The nature of the immunity induced by i.n. immunization of PrV DNA vaccine did not provide effective protection against i.n. challenge of a virulent PrV strain. Therefore, although i.n. immunization was a useful route for inducing mucosal immunity at the entry site of PrV, i.n. immunization provided no effective protection against PrV infection.

One aim of mucosal antigen administration is to exploit the well-known common mucosal defense mechanisms and to induce barrier levels of immunity at multiple mucosal surfaces. Moreover, a particular advantage of i.n. vaccine delivery is that this route requires less antigen than the oral immunization route. This is an important concept for preventing invasion by pathogens that enter the body *via* mucosae and damage the mucosal site. Here, we apparently showed that mucosal delivery of PrV DNA vaccine engaged the common mucosal immune system and induced detectable immunity at a distal mucosal site. However, with regard to defense against a virulent virus, the level of protection induced by i.n. administration of PrV vaccine was less than those induced by i.m. immunization of PrV DNA vaccine, and was clearly not a barrier to viral invasion. Hence, even animals with readily detectable PrV-specific IgA responses succumbed to a virulent PrV infection. As was documented previously, immunity to PrV may depend more on the function of the T-cell defense system in the infected tissues than on the function of humoral antibodies, especially mucosal IgA, in mucosal tissues [2, 8, 35]. The observation that immunity against human herpes virus was similar in both IgA-knockout and wild-type mice also supports this conclusion [31]. On the other hand, some reports of studies using a passive antibody transfer approach indicate that protection against PrV infection can be mediated by humoral immunity [17, 26]. Therefore, both cell-mediated immunity, especially Th1-biased immunity, and antibodies may play critical roles in protecting against PrV infection.

The notion described above is also supported by our observation that i.m. immunization of PrV DNA vaccine induced Th1-biased immunity, which subsequently conferred effective protective immunity against a virulent PrV infection. In contrast, i.n. delivery of PrV DNA vaccine enhanced production of the Th2-type cytokine IL-4 from stimulated CD4+ T cells, indicating that mucosal delivery of PrV DNA vaccine drove more Th2-biased immunity than systemic delivery did. Conceivably, the altered nature of the immunity and weak responses induced by i.n. immunization might make immunized animals more susceptible to PrV infection [2, 8, 35]. In particular, IFN-γ produced from mainly Th1-type CD4+ T cells may indirectly ensure that the immune system is able to react instantly and promptly by protecting APC from viral infection [25]. IFN-γ also plays an important role in the virus-induced IgG2a response [1]. Using a murine model, Schijns *et al.* [32] showed that vaccination against PrV induces an IFN-γ-dependent, anti-PrV IgG2a response. These anti-PrV IgG2a antibodies can transfer their protection to naïve recipient mice [32]. Our previous results also support such findings [38–40]. Moreover, PrV gB is a very important antigen for inducing Th1-biased immunity and conferring effective protection against virulent viral infection [39]. In the present study, mucosal delivery of plasmid DNA encoding gB altered the induced immunity to Th2-type, which could be detrimental in conferring effective protection against intracellular virus infection [2, 8, 35]. However, the mucosal route for immunization of a DNA vaccine has

several advantages in that it is noninvasive, safe, comfortable, and not painful. Therefore, to improve the usefulness of mucosal DNA vaccine delivery, strategies for modulating and enhancing immunity from the DNA vaccine are needed when considering the application of PrV DNA vaccine to the natural hosts, the pigs. In efforts to improve the efficacy of mucosal delivery, Sindbis-derived plasmid vector [5, 6], genetic incorporation of Th1-type inducing cytokines [38, 40], and modifying protocols of immunization, such as heterologous prime-boost vaccination [34], could be considered.

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