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Multiple Alternating Immunizations with DNA Vaccine and Replication-incompetent Adenovirus Expressing gB of Pseudorabies Virus Protect Animals Against Lethal Virus Challenge

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The prime-boost vaccination with DNA vaccine and recombinant viral vector has emerged as an effective prophylactic strategy to control infectious diseases. Here, we compared the protective immunities induced by multiple alternating immunizations with DNA vaccine (pCIgB) and replication-incompetent adenovirus (Ad-gB) expressing glycoprotein gB of pseudorabies virus (PrV). The platform of pCIgB-prime and Ad-gB-boost induced the most effective immune responses and provided protection against virulent PrV infection. However, priming with pCIgB prior to vaccinating animals by the DNA vaccine-prime and Ad-boost protocol provided neither effective immune responses nor protection against PrV. Similarly, boosting with Ad-gB following immunization with DNA vaccine-prime and Ad-boost showed no significant responses. Moreover, whereas the administration of Ad-gB for primary immunization induced Th2-type-biased immunity, priming with pCIgB induced Th1-type-biased immunity, as judged by the production of PrV-specific IgG isotypes and cytokine IFN-γ. These results indicate that the order and injection frequency of vaccine vehicles used for heterologous prime-boost vaccination affect the magnitude and nature of the immunity. Therefore, our demonstration implies that the prime-boost protocol should be carefully considered and selected to induce the desired immune responses.

Keywords: Pseudorabies virus (PrV), prime-boost vaccination, DNA vaccine, replication-incompetent adenovirus

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 influential infectious diseases in the swine industry [18]. Similar to human alphaherpesvirus, PrV establishes a lifelong infection in a variety of nervous tissues of the natural host, and this infection can be reactivated through experimental or natural stresses [43]. Various vaccination strategies have been employed to control the outbreak of AD using modified live or inactivated vaccines [17, 22]. The modified live vaccines (MLVs), which generally induce long-lasting immunity, carry a risk of insufficient attenuation and/or genetic instability. Inactivated vaccines also are less efficient and require repeated doses. Hence, vaccination with the plasmid DNA encoding glycoprotein antigens of PrV has been one of the most significant advances in PrV vaccines [6, 7, 9, 39, 41]. Generally, a combination of plasmid DNAs expressing the three major glycoproteins (gB, gC, and gD), which are involved in the essential steps of viral infection, was investigated [9, 41]. Among these three major glycoproteins, plasmid DNA encoding gB has been reported to induce the strongest cell-mediated immunity and to reduce the level of viral excretion after a challenge infection [9]. However, despite the significant progress made, the immunogenicity of PrV DNA vaccines requires considerable improvement owing to the high mortality rate from the disease in vaccinated animals [6, 7, 9, 18, 39, 41].

Recently, multiple prime-boost vaccinations with alternative vaccine vehicles expressing the same antigen have emerged as an effective strategy for eliciting robust immune responses to the target antigen [5, 14, 24]. With this prime-boost strategy, greater outcomes of immunity can be synergistically established compared with a single vaccine administration or homologous boost strategies [14]. In such strategies, the prime-boost protocol, in which antigen-encoding DNA vaccine is administered first, followed by a boost with recombinant viral vector expressing the same antigen, has

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elicited effective protective immunity in both mouse and primate models of several infectious diseases [1, 19, 21]. Moreover, the magnitude of the cellular immune response following the recombinant viral boost has been shown to correlate with the initial response following the priming injection with plasmid DNA encoding the antigen [32]. Thus, enhancing the immunogenicity of the initial priming vaccination might substantially affect the magnitude of the immune response following the recombinant viral boost.

Various viral vectors expressing foreign antigen, such as modified vaccinia virus Ankara (MVA), adenovirus (Ad), or Fowlpox, have been used for the booster after priming with DNA vaccine [16, 32, 33]. DNA- and vaccinia-based vaccines for a pre-erythrocytic malaria antigen that were delivered in a prime-boost protocol induced 5- to 10-fold greater T-cell responses than each vaccine alone [30]. In addition, gene-based vectors, such as replication-incompetent adenovirus, have proven particularly effective in eliciting enhanced cellular and humoral immunities compared with either agent alone [5, 28, 40]. Because Ad infects a broad spectrum of mammalian cells including dendritic cells (DCs) [2, 27, 34], it may enhance the immune responses induced by different priming vectors such as DNA vaccine. In this investigation, to compare the protective immunity induced by multiple immunizations with DNA vaccine and replication-incompetent adenovirus expressing PrV gB, we designed several prime-boost protocols. Among the prime-boost protocols, the platform of DNA vaccine-prime and Ad-boost elicited effective Th1-type-biased protection against virulent PrV challenge. Interestingly, however, another priming with DNA vaccine or another boosting with recombinant adenovirus provided no significant protective immunity. Therefore, these results suggest that the order and injection frequency of vaccine vehicles used for prime-boost vaccination should be carefully considered to induce the desired immune responses.

MATERIALS AND METHODS

Animals

Female BALB/c (H-2^b) mice, 5 to 6 weeks of age, were purchased from Damul Science Inc. (Daejeon, 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) 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). PK-15 cultures were infected with 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 adsorption, the inoculum was removed, and 10 ml of maintenance medium containing 2% FBS was added. Approximately 48–72 h post-infection, host cell cultures that exhibited 80–90% cytopathic effect (CPE) were harvested. The virus stocks were concentrated by centrifugation at 50,000 ×g, titrated by plaque assays, and stored in aliquots at −80°C until needed.

Plasmid DNA Preparation

Plasmid DNA encoding gB of PrV under the control of the cytomegalovirus (CMV) promoter (pCIgB) has been described in detail elsewhere (Fig. 1A) [9]. For immunization, plasmid DNA was purified by polyethylene glycol precipitation as described previously [8, 24]. 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

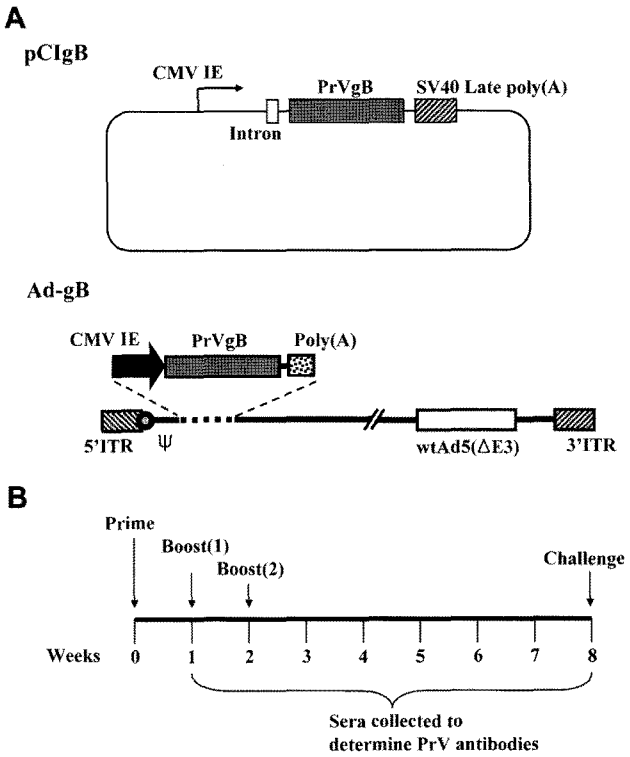

Fig. 1. Constructs used for prime-boost immunization (**A**), and immunization schedule (**B**).
A. Schematic showing the DNA vaccine and replication-incompetent adenovirus vector expressing PrV gB. CMV IE: cytomegalovirus immediate early promoter; Poly(A): SV40 polyadenylation signal; ITR: inverted terminal repeat; ψ: packaging signal. **B**. Mice were primed with either 100 μg of pCIgB or 10^6 PFU of Ad-gB *via* i.m. route and then boosted 7 days later with alternate vaccine vehicle *via* the same route [Boost(1)]. For the second boost [Boost(2)], some mice were re-injected i.m. with alternate vaccine vehicle 7 days later.

Limulus amebocyte lysate (LAL) test (<0.05 EU/μg). The *in vivo* effect of endotoxin and CpG was addressed by parallel administration of the control vector, pCI-neo (Promega, Madison, WI, U.S.A.).

Construction and Purification of the Recombinant Adenovirus

The E1- and E3-deleted expression vector into which PrVgB is cloned was used to construct replication-incompetent adenovirus expressing glycoprotein gB of PrV [3, 36]. PrV gB was expressed in the replication-incompetent adenovirus by cloning the PrV gB gene under the control of the human CMV promoter (Fig. 1A). We initially constructed recombinant entry vectors pENTR11 (Invitrogen) containing the PrVgB gene by PCR amplification and subcloning. Using LR Clonase (Invitrogen) for catalysis, the recombinant entry vectors pENTR11 containing the gB gene were mixed with adenoviral destination vector DNA, pAd/CMV/V5-DEST (Invitrogen), to generate recombinant adenoviral DNA plasmids containing the PrVgB gene. After transforming the recombinant adenoviral plasmid DNA into competent *E. coli*, we extracted and purified DNA from selected putative positive clones identified by PCR amplification and electrophoretic detection. Those putative clones were also cultured on LB plates containing 30 μg/ml chloramphenicol, since true expression clones would be ampicillin-resistant and chloramphenicol-sensitive. Following digestion of the recombinant adenoviral plasmid DNA containing the gB gene with the restriction enzyme Pac I, human embryonic kidney 293A cells were transfected to generate replication-incompetent adenovirus. Culture medium was replaced with fresh complete culture medium every 2–3 days until visible regions of CPE were observed. When approximately 50–70% CPE was observed, adenovirus-containing cells and media were harvested. The replication-incompetent adenoviruses expressing gB (Ad-gB) were purified with the Adeno-X mini purification kit (Clontech, Mountain View, CA, U.S.A.), titrated by plaque assays, and stored at −80°C until use. The expression of the gB gene was identified with reverse transcriptase (RT)-PCR after NIH3T3 cells were infected with Ad-gB.

Protocol for Vaccination and Sample Collection

Groups of mice (5- to 6-wk-old female mice) were immunized with either 100 mg of pCIgB or 10^6 PFU of Ad-gB *via* the intramuscular (i.m.) route and then boosted 7 days later with alternative vaccine vehicle *via* the same route. The i.m. immunization was performed by injecting the indicated immunogen into the anterior tibialis muscle. For the second boost, some boosted mice were re-injected i.m. with alternate vaccine vehicle 7 days later (Fig. 1B). Control mice were given replication-incompetent adenovirus expressing the LacZ gene (Ad-LacZ) and empty plasmid DNA vector, pCI-neo. Serum samples were collected on the seventh day after each immunization by retroorbital bleeding and stored at −80°C until needed.

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

A standard enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of PrV-specific antibodies in the serum samples. Briefly, ELISA plates were coated overnight at 4°C with an optimal dilution (0.5–1.0 μg/well) of semipurified PrV antigen in the sample wells and goat anti-mouse IgG/IgG2a/IgG1 (Southern Biotechnology Associate Inc., Birmingham, AL, U.S.A.) in the standard wells. The viral antigen for coating was prepared by semipurification 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.) [11]. The plates were washed three times with PBS-Tween 20 (PBST) and blocked with 3% nonfat dehydrated milk. The samples were serially diluted 2-fold, and incubated for 2 h at 37°C. This was followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG/IgG2a/IgG1 for 1 h. 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.).

Cytokine IFN-γ ELISA Following *In Vitro* Stimulation of CD4+ T Cells

Six weeks after the final immunization, the mice were sacrificed to prepare splenocytes. 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. The remaining cells were used as responder cells. The enriched antigen-presenting cell (APC) populations, which were obtained as described previously [8], 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 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 for 48 h as a polyclonal positive stimulator.

ELISA was used to determine cytokine IFN-γ levels in the culture supernatants [15]. The ELISA plates were coated with IFN-γ anti-mouse antibodies (Pharmingen, San Diego, CA, U.S.A.; Clone No. R4-6A2) 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 recombinant IFN-γ protein (Pharmingen) were added to the plates and incubated overnight at 4°C. Biotinylated IFN-γ antibody (Pharmingen; Clone No. XMG1.2) was 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. Cytokine concentration was determined using an automated ELISA reader.

Virus Challenge Experiment

Six weeks after the final immunization, the immunized mice were infected i.n. with the virulent PrV Yangsan strain (10 LD_{50}). The challenged mice were examined daily to assess signs of inflammation, illness, and death. 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.

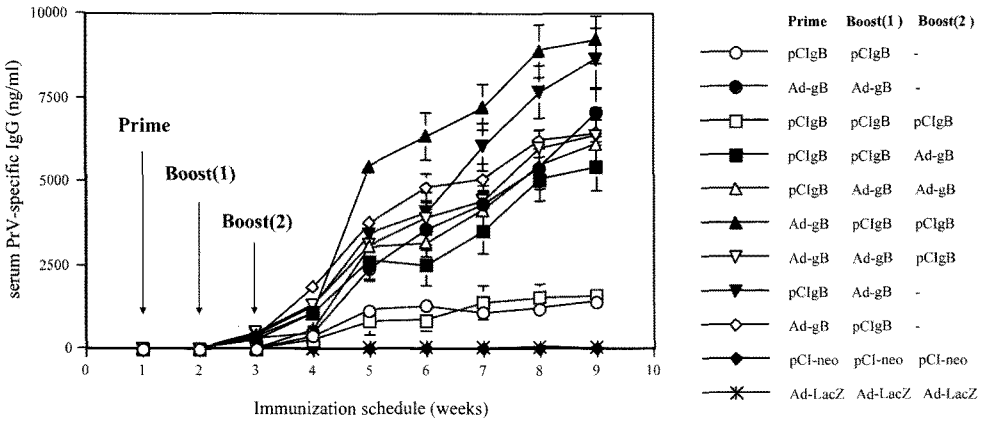

Fig. 2. Serum PrV-specific IgG levels of animals immunized with multiple prime-boost protocols.
Groups of mice were immunized primarily with either 100 μg of pCIgB or 10^6 PFU of Ad-gB *via* i.m. route (Prime) and then boosted 7 days later with alternate vaccine vehicle *via* the same route [Boost(1)]. For the second boost [Boost(2)], some mice were re-injected i.m. with alternate vaccine vehicle 7 days later. The PrV-specific IgG levels in the sera were determined by ELISA weekly following primary immunization. The graphs represent the average and standard deviation (SD) from 7 mice per group.

RESULTS

Systemic Antibody Responses Induced by Multiple Immunizations with DNA Vaccine and Recombinant Adenovirus

The aim of this study was to compare the protective immunities elicited by multiple prime-boost protocols. This prime-boost protocol used alternating i.m. administration of DNA vaccine and replication-incompetent adenovirus expressing PrVgB. We first assessed the humoral immune responses following multiple prime-boost protocols. Groups of mice were primed i.m. with either pCIgB or Ad-gB and subsequently boosted 7 days later either with the same vaccine or with the alternate vaccine *via* the i.m. route. Some boosted mice were immunized i.m. with the alternate vaccine 7 days later, as the second boost. The levels of total PrV-specific IgG in sera were monitored weekly until 6 weeks after the last immunization. As shown in Fig. 2, multiple alternating immunizations with pCIgB and Ad-gB were categorized into three patterns, based on the induced levels of PrV-specific IgG in sera (high, medium, and low). Among the several prime-boost protocols, groups of mice that received Ad-gB and then pCIgB for the first and second boosts, respectively, had the most potent IgG responses (closed triangle), whereas multiple injections of DNA vaccine, pCIgB, induced weak PrV-specific IgG responses in sera (open circle and square). Interestingly, mice primed with pCIgB and then boosted with Ad-gB had comparable levels of PrV-specific IgG to mice primed with Ad-gB and boosted twice with pCIgB (reversed closed triangle) (Fig. 2). Moreover, the PrV-specific responses induced by priming with pCIgB and boosting with Ad-gB were not enhanced by secondary boosting with Ad-gB (open triangle). Also noteworthy, lower PrV-specific IgG responses were elicited in mice that received pCIgB for both the prime and the first boost and Ad-gB for the second boost than in mice given pCIgB-prime and Ad-gB-boost (closed square). These results indicate that the order and injection frequency of vaccine vehicles used for prime and boost should be carefully considered to induce the optimal final immune response.

When the distribution of the PrV-specific IgG isotypes (IgG2a and IgG1) induced by alternating multiple immunizations with pCIgB and Ad-gB was determined, the patterns of isotype levels were shown to depend on the injection order of vaccine vehicles used for prime and boost (Fig. 3). Animals given Ad-gB-prime and pCIgB-boost (1 and 2) produced higher amounts of PrV-specific IgG1 than groups given pCIgB-prime and Ad-gB-boost, resulting in a low IgG2a/IgG1 ratio. In contrast, animals primed with pCIgB and boosted with Ad-gB showed the highest production of IgG2a isotype, indicating that this prime-boost protocol induced Th1-type-biased immunity. Overall, primary immunization with Ad-gB induced higher production of PrV-specific IgG1 isotype, indicating that priming with PrVgB-expressing recombinant adenovirus mounted Th2-type-biased immunity (middle graph of Fig. 3). However, higher ratios of IgG2a/IgG1 were observed if the DNA vaccine, pCIgB, was used for prime (lower graph of Fig. 3). Therefore, this finding indicates that the type of vaccine vehicle used for prime could determine the nature of immunity induced by the booster.

Pattern of Cytokine IFN-γ Produced from Stimulated CD4+ T Cells

To further evaluate the nature of immunity induced by alternating multiple immunizations with pCIgB and Ad-gB, we determined the levels of the major Th1-type cytokine (IFN-γ) produced from CD4+ T cells stimulated with

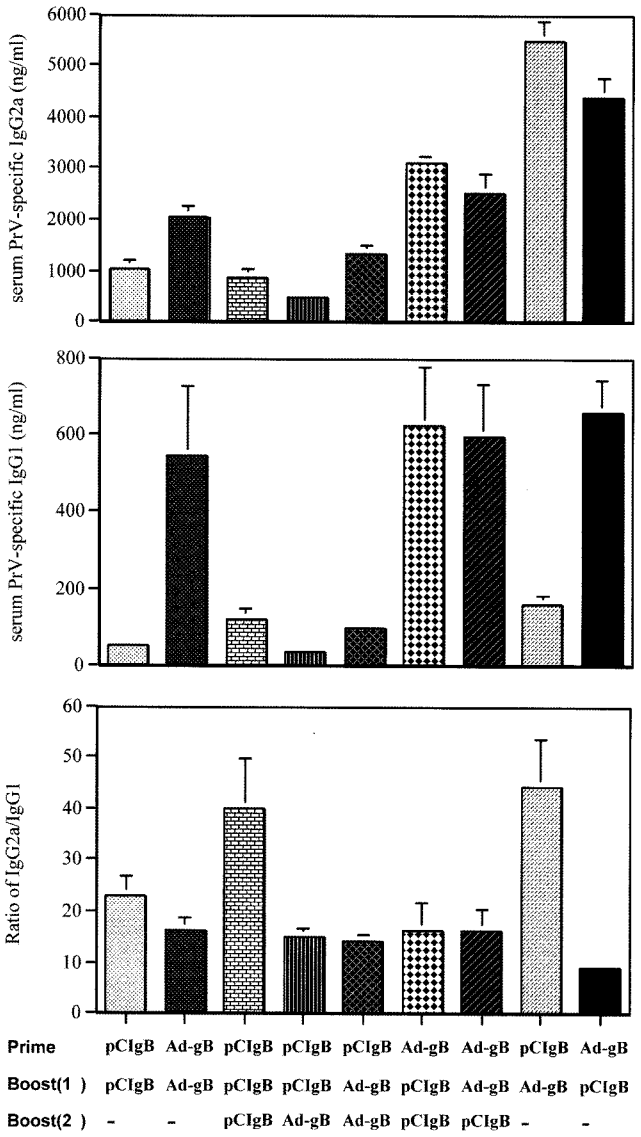

Fig. 3. Distribution of serum PrV-specific IgG isotypes (IgG1 and IgG2a) in animals immunized with multiple prime-boost protocols.

Groups of mice were immunized primarily with either 100 μg of pCIgB or 10^6 PFU of Ad-gB *via* i.m. route (Prime) and then boosted 7 days later with alternate vaccine vehicle *via* the same route [Boost(1)]. For the second boost [Boost(2)], some mice were re-injected i.m. with alternate vaccine vehicle 7 days later. The levels of PrV-specific IgG isotypes (IgG2a and IgG1) in the sera were determined by ELISA six weeks after the final immunization. Each bar represents the average and standard deviation (SD) from 7 mice per group.

antigen protein, which is known to induce the predominant expansion of immune CD4+ T cells [27]. As shown in Fig. 4, animals that were primed with pCIgB and subsequently boosted with Ad-gB, which was shown to elicit Th1-type-biased immunity, produced the highest amount of IFN-γ from stimulated CD4+ T cells. However, animals primed with Ad-gB and then boosted twice with pCIgB, which induced the most potent responses of total PrV-specific

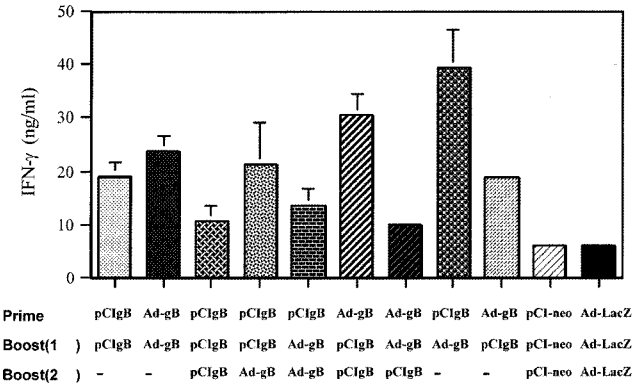

Fig. 4. The production of cytokine IFN-γ from splenocytes of animals immunized with multiple prime-boost protocols.

Six weeks after the final immunization, the responder cells were mixed with irradiated syngeneic enriched APCs that had been pulsed with UV-inactivated PrV and then incubated for 3 days. IFN-γ levels in the supernatants of the stimulated T cells were determined by cytokine ELISA. The test was carried out in quadruplicate wells. Each bar represents the average and SD from three independent experiments.

IgG, produced significantly less IFN-γ than mice primed with pCIgB and boosted with Ad-gB (p=0.045). Interestingly, animals that received pCIgB for prime and the first boost and Ad-gB for the second boost produced less IFN-γ. Moreover, another boost with Ad-gB did not enhance IFN-γ production in animals primed with pCIgB and boosted with Ad-gB. These results, together with the humoral responses, indicate that the order and injection frequency of vaccine vehicles used for prime and boost could affect the nature of the humoral and cellular immunities. Therefore, the order and types of vaccine vehicles should be carefully selected to induce the desired nature of immunity.

Protective Immunity Against Lethal Virus Challenge

We subsequently determined the protective immunity induced by alternating multiple immunizations with pCIgB and Ad-gB. To compare the protective efficacy of immunity afforded by multiple prime-boost vaccinations, groups of mice immunized by several prime-boost protocols were challenged i.n. with a highly virulent PrV YS strain (10 LD_{50}) six weeks after the last immunization. Initially, when the levels of anamnestic PrV-specific IgG in sera were determined 3 days after the challenge, the highest levels of serum IgG antibodies were observed in mice that received pCIgB for prime and Ad-gB for boost (Fig. 5). This anamnestic level of serum IgG antibody afforded by priming with pCIgB and boosting with Ad-gB was significantly higher than that in mice primed with Ad-gB and subsequently boosted with pCIgB twice (p=0.039). Moreover, animals that received pCIgB for prime and Ad-gB for boost showed the most effective protection against virulent viral challenge (survival rate=43%) (Fig. 6). Priming with Ad-gB and subsequent boosting with pCIgB twice provided less effective

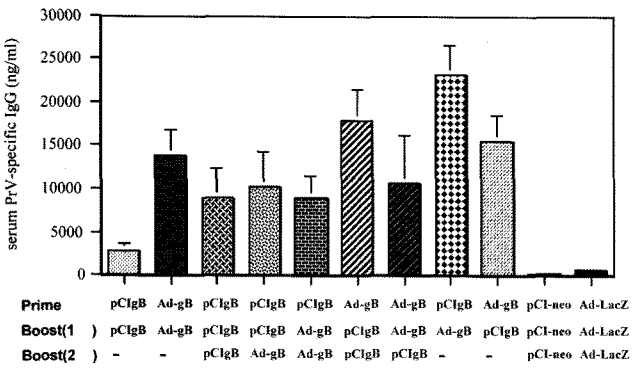

Fig. 5. Anamnestic PrV-specific IgG responses determined 3 days after challenge with virulent PrV.
Six weeks after the final immunization, groups of mice (n=7) immunized with multiple prime-boost protocols were challenged i.n. with PrV YS strain (10 LD_{50}), and the sera were collected 3 days later. The levels of PrV-specific IgG in the sera were determined using conventional ELISA. Each bar represents the average and SD per group.

protection against a virulent viral challenge (survival rate= 28%). Mice immunized with other prime-boost protocols showed no significant protection against virulent PrV challenge. Therefore, these results suggest that priming with DNA vaccine and subsequent boosting with PrVgB-expressing recombinant adenovirus provide effective protective immunity against PrV challenge. However, addition of either another priming with pCIgB or another boosting with Ad-gB to the pCIgB-prime and Ad-gB-boost protocol provided no effective protection against viral challenge.

DISCUSSION

Vaccination protocols commonly require multiple immunizations to achieve robust, protective, and sustained immune responses. In particular, heterologous prime-boost vaccination with DNA vaccine and recombinant viral vector has emerged as an effective strategy for eliciting a robust response to target antigen [5, 14, 24]. In such vaccination strategies, the most effective approach has proven to be priming with plasmid DNA and boosting with recombinant viral vector expressing the same antigen [1, 19, 21]. This approach has been used extensively in the development of vaccines against a number of pathogens including HIV, herpesvirus, hepatitis C virus, Ebola virus, and Venezuelan equine encephalitis virus [1, 5, 26, 28]. In addition, the results described in the present study support that the platform of DNA vaccine-prime and recombinant adenovirus-boost induces effective immune responses. Of particular interest, priming with the DNA vaccine, pCIgB, prior to vaccination with DNA vaccine-prime and Ad-boost protocol, provided no effective immune response or protection against PrV. Similarly, boosting with replication-incompetent adenovirus expressing gB (Ad-gB) following immunization with the DNA vaccine-prime and Ad-boost protocol did not enhance the immune responses. Moreover, whereas the administration of recombinant adenovirus for primary immunization induced Th2-type-biased immunity, priming with DNA vaccine resulted in Th1-type-biased immunity. These findings suggest that the order and injection frequency of vaccine vehicles used for heterologous prime-boost vaccination affect the magnitude and nature of immunity.

Heterologous prime-boost immunization is known to confer synergistically stronger responses to antigens and greater protection than immunization with either vaccine alone [5, 14, 24]. However, the immunological basis for this outcome remains to be resolved. The success of this approach may depend on several factors. In some instances, immune responses to repeated administration of the vector

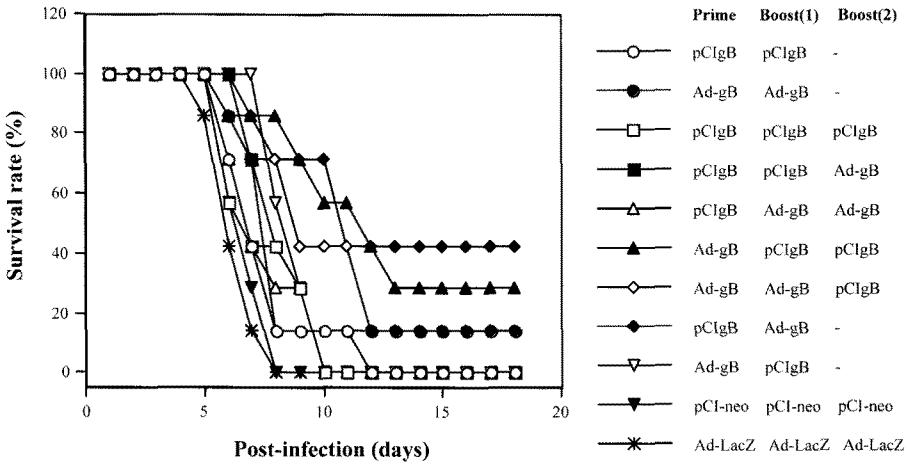

Fig. 6. Susceptibility of animals immunized with multiple prime-boost protocols to virulent PrV.
Six weeks after the final immunization, groups of mice (n=7) immunized with multiple prime-boost protocols were challenged i.n. with PrV YS strain (10 LD_{50}). The challenged mice were examined daily for any signs of inflammation, illness, and death until 18 days post-challenge.

used for the primary immunization can neutralize the immunity induced by a booster, subsequently reducing the effective responses. The prime-boost vaccination may also allow for alternative modes of antigen presentation, depending on the diversity of gene delivery. In particular, boosting with recombinant viral vector may selectively expand the small population of antigen-specific CD4/CD8 T cells by inducing type I INF production, leading to IL-15 production, which has been known to maintain CD8+ T cell proliferation and survival [4, 38, 42]. Moreover, Ad appears to infect early DCs, which may differentiate to mature DCs that more effectively present antigens [5, 28, 40]. Ad also synthesizes larger quantities of proteins that are taken up by endocytosis. Thus, the divergent cell targeting and antigen presentation complement each other, allowing a greater outcome of immune responses than with either vaccine vehicle alone. However, why the priming with DNA vaccine prior to immunization with the DNA vaccine-prime and Ad-boost protocol or secondary boosting with Ad after immunizing with the prime-boost protocol provided no effective immune responses is not fully understood. Such triple immunizations with pCIgB and Ad-gB elicited less immune responses and protection than the DNA vaccine-prime and Ad-boost regimen. Conceivably, optimally induced immunity by the primary immunization may be needed to induce greater immune responses following prime-boost vaccination.

Another point of interest is that Th1-type-biased immunity was observed if the DNA vaccine was used for prime, resulting in more effective protection against virulent PrV infection. In contrast, recombinant adenovirus for prime induced higher production of IgG1 isotype, which represents Th2-type-biased immunity. This suggests that the nature of the immunity induced by prime-boost vaccination could be modulated by the order of administration of the vaccine vehicles used. In particular, the initial priming vaccination appears to substantially affect the nature of the immune responses following prime-boost vaccination since DNA vaccine has been known to induce Th1-type-biased immunity with a encoded CpG motif [31, 35]. Moreover, the Th1-type-biased immunity induced by prime-boost vaccination provided effective protection against virulent PrV infection. In reality, the significance of Th1-type CD4+ effector T cells in protective immunity was shown using a murine PrV-infected model [9, 10, 13]. Conceivably, T-cell-mediated immunity might be a more important parameter for conferring early protection against a challenge, with a close correlation between the magnitude of IFN-γ production and the involvement of Th1-type CD4+ T cells [10, 11]. IFN-γ may indirectly ensure the ability of the immune system to react instantly by protecting the APC from a viral infection [20]. IFN-γ also plays an important role in the virus-induced IgG2a response [29], as shown by the observation that vaccination against PrV induces IFN-γ-dependent, anti-PrV IgG2a responses [29]. These anti-PrV IgG2a antibodies can transfer their protection to naïve recipient mice [29]. Furthermore, the present study found a close correlation between the anamnestic IgG levels determined 3 days after a challenge and the rate of protection against virulent PrV infection, although the level of IgG2a isotype was not measured. Therefore, Th1-type-biased, cell-mediated, and anti-PrV humoral immunity is believed to be an important protective effector mechanism against PrV infection.

The presented prime-boost vaccination can be regarded as an effective prophylactic strategy to control the outbreak of Aujeszky's disease. Furthermore, compared with the application of conventional vaccines, such as modified live and inactivated vaccines, the use of DNA vaccine for prime is expected to avoid interference by maternal antibodies [12, 37]. Discrimination between vaccinated and field-infected animals is also possible owing to the use of specific glycoprotein gB only. Moreover, in this particular application, the recombinant adenovirus is used as a booster to vaccinate neonates quickly after birth, independent of the presence or absence of maternal antibodies [23, 44]. Thus, in veterinary applications for pigs and other animals that are slaughtered a few months after birth, very young pigs (1 day of age) can be successfully vaccinated by effective prime-boost protocols without the worry of vaccine failure due to interference by maternal antibodies.

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