Polarization of protective immunity induced by replication-incompetent adenovirus expressing glycoproteins of pseudorabies virus

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Abbreviations: Ad, adenovirus; CMV, cytomegalovirus; CPE, cytopathic effect; i.n., intranasal; ITR, inverted terminal repeat; LN, lymph node; PrV, pseudorabies virus; rAd, recombinant adenovirus

Abstract

Replication-incompetent adenoviruses expressing three major glycoproteins (gB, gC, and gD) of pseudorabies virus (PrV) were constructed and used to examine the ability of these glycoproteins to induce protective immunity against a lethal challenge. Among three constructs, recombinant adenovirus expressing gB (rAd-gB) was found to induce the most potent immunity biased to Th1-type, as determined by the IgG isotype ratio and the profile of the Th1/Th2 cytokine production. Conversely, the gC-expressing adenovirus (rAd-gC) revealed Th2-type immunity and the gD-expressing adenovirus (rAd-gD) induced lower levels of IFN-γ and IL-4 production than other constructs, except IL-2 production. Mucosal delivery of rAd-gB induced mucosal IgA and serum IgG responses and biased toward Th2-type immune responses. However, these effects were not observed in response to systemic delivery of rAd-gB. In addition, rAd-gB appeared to induce effective protective immunity against a virulent viral infection, regardless of whether it was administered via the muscular or systemic route. These results suggest that administration of replication-incompetent adenoviruses can induce different types of immunity depending on the expressed antigen and that recombinant adenoviruses expressing gB induced the most potent Th1-biased humoral and cellular immunity and provided effective protection against PrV infection.

Keywords: adenoviruses, human; herpesvirus 1, Suid; Th1 cells; Th2 cells; vaccination

Introduction

Adenovirus (Ad) is double-stranded linear DNA viruses with genomes of approximately 36 kb in length. Ad is used in the expression of genes of interest for gene therapy and vaccine development (Shiver et al., 2002; Mizuguchi, 2004; Plog et al., 2006; Khanam et al., 2007). In one such method, foreign genes are cloned into regions that are dispensable for viral replication, such as the E3 gene, or into the intersection between E4 and the right inverted terminal repeat (ITR) (Babiuk et al., 2000), which results in replication-competent viruses. However, the biosafety of such viruses remains to be addressed. In another method, a large deletion in the E1 and E3 genes was employed to create a replication-incompetent adenovirus. Such replication-incompetent adenoviruses can grow only in cells that contain the complement E1 region of the adenovirus genome (Babiuk et al., 2000), and this increases their biosafety for experimental use. Also, high levels of expression are achieved in replication-incompetent adenoviruses when the foreign genes are under the control of constitutive promoters such as the CMV promoter (Ambriovic

et al., 1997). Other advantages of recombinant adenoviruses include their broad host range and, in the case of livestock, the lack of maternally derived antibodies that interfere with vaccine efficacy in young and growing animals (Monteil *et al.*, 1997).

The pseudorabies virus (PrV) is a porcine alphaherpesvirus that causes the fatal Aujeszky's disease in swine. Aujeszky's disease is an important infectious disease in the swine industry (Kluge *et al.*, 1999), and control of disease outbreaks in swine by immunization with active, modified live, and inactivated vaccines have been attempted (Kit, 1988; Mettenleiter, 1996). Attenuated vaccines, which generally induce long-lasting immunity, carry a risk of insufficient attenuation and genetic instability. Inactivated vaccines are less efficient than attenuated vaccines and require repeat doses. Therefore, the use of live recombinant vaccines carrying individual PrV genes may provide a safe alternative to the use of attenuated live vaccine strains.

Three major glycoproteins (gB, gC, and gD) are involved in the essential steps in the progress of PrV infection because of their roles in the induction of protective immune responses against virus infection, as noted in vaccination experiments with mouse or pig models (Monteil *et al.*, 1997; van Rooij *et al.*, 1998; Hong *et al.*, 2002; Yoon *et al.*, 2006). Several B-cell epitopes detected on PrV gB and gC glycoproteins (Ober *et al.*, 1998; Zaripov *et al.*, 1998, 1999; Ober *et al.*, 2000) and T-cell epitopes detected on the PrV gC glycoprotein (Ober *et al.*, 1998; Ober *et al.*, 2000) induce both humoral and cytotoxic responses (van Rooij *et al.*, 1998, 2000). Additionally, vaccination of mice or pigs with either purified recombinant gD or recombinant gD-expressing virus vectors conferred protection to the animals (Eloit *et al.*, 1995; Gonin *et al.*, 1996; Monteil *et al.*, 1997, 2000). However, the relative potency of recombinant viral vectors expressing the three major glycoproteins has not yet been investigated. Therefore, we constructed replication-incompetent adenoviruses expressing these gB, gC, and gD, and examined their ability to induce immune responses against PrV. Among three recombinant adenovirus constructs, the gB-expressing adenovirus was found to induce the most potent Th1-type immunity. Further, we investigated the nature of the protective immunity induced by systemic and mucosal delivery of a replication- incompetent gB-expressing adenovirus.

Materials and Methods

Animals

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

Cells and viruses

The wild-type pseudorabies virus (PrV) Yangsan (YS) strain was generously supplied by the National Veterinary Research and Quarantine Service of the Republic of Korea. PrV was propagated in a porcine kidney cell line, PK-15, using DMEM supplemented with 2.5% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). The PK-15 cultures were infected with PrV at a multiplicity of infection (MOI) of 0.01, and then incubated in a humidified CO$_2$ incubator for 1 h at 37°C. The inoculum was removed after adsorption and 10 ml of a maintenance medium containing 2.5% FBS was added. Cultures of host cells showing 80-90% cytopathic effect (CPE) were harvested at approximately 48 to 72 h post-infection. Virus stocks were concentrated by centrifugation at 50,000 g, titrated by a plaque assay, and stored in aliquots at -80°C until needed.

Construction of replication-incompetent adenoviruses expressing glycoproteins

The Gateway cloning technique was used to construct replication-defective adenoviruses expressing PrV glycoproteins (Walhout *et al.*, 2000; Suzuki *et al.*, 2005). We constructed recombinant entry vectors (pENTR; Invitrogen, Carlsbad, CA) containing PrV glycoprotein genes by PCR amplification. Specific primers were used to amplify the gB, gC, and gD genes of the PrV as described in Table 1. Each fragment of the glycoprotein gene was amplified in 50 μl of a PCR buffer [2 mM each primer, 1 mM MgCl$_2$, 100 μM each deoxynucleoside triphosphate, 2.5 U of *i*-pfu DNA polymerase

Table 1. Sequences of the primers used for construction of replication-defective adenovirus expressing glycoproteins.

Construc-tion		Primers for construction of rAd	
		Sequences (5'→3')	Product size (bp)
rAd-gB	F	CTTCAGGTCCGTCTTCCACTG	2901
	R	GCATCTTTATTGTTTCCCGCG	
rAd-gC	F	AAATCCGTTTCCTGATTCACG	1615
	R	CGTTTATTGATTCGACGTGGG	
rAd-gD	F	CCTTAAAATTGGGTCGGCGTC	1323
	R	ATCATCATCGACGCCGGTACT	

(iNtRON Biotech, Daejeon, Korea), 100 ng PrV genome DNA as template]. The first cycle was performed at 96°C for 3 min, 58°C for 30 s, and 68°C for 3 min. The DNA was then amplified for 34 cycles (94°C for 1 min, 58°C for 30 s, 68°C for 3 min) followed by a final extension step at 72°C for 10 min. The recombinant entry vector pENTR11 containing each glycoprotein gene was mixed with the adenoviral destination vector (pAd/CMV/V5-DEST; Invitrogen) to generate the recombinant adenoviral DNA plasmid containing PrV glyco-protein genes in the presence of LR Clonase (Invitrogen) for catalysis. The recombinant adeno-viral plasmid DNA was transformed into competent *E. coli* and putative positive clones were selected by M13 primer-PCR amplification and detection by electrophoresis. Those putative clones were also cultured on LB plates containing 30 μg/ml chlo-ramphenicol since true expression clones would be ampicillin-resistant and chloramphenicol-sensitive. Following digestion of recombinant adenoviral plasmid DNAs with the *Pac* I restriction enzyme, human embryonal kidney 293A cells were trans-fected to generate replication-incompetent adeno-viruses expressing gB (rAd-gB), gC (rAd-gC), and gD (rAD-gD). Viruses were then purified using an Adeno-X mini purification kit (Clontech, Mountain View, CA), titrated by plaque assay, and stored at -80°C until use. Recombinant viruses (rAd-gB, rAd-gC, and rAd-gD) were used to infect NIH3T3 cells, and the expression of each glycoprotein was identified by immunoblot using polyclonal anti-bodies.

Immunization and sample collection

Groups of female mice were immunized with replication-incompetent adenoviruses expressing PrV glycoproteins (rAd-gB, rAd-gC, and rAd-gD) by either the intranasal (i.n.) or intramuscular (i.m.) route. For i.m. administration, recombinant adeno-viruses (10^6 pfu/mouse) were injected into the anterior tibialis muscle three times at weekly intervals (0, 7, and 14 days). The i.n. immuniza-tions were also performed three times at weekly intervals (0, 7, and 14 days) by depositing 10^6 pfu of recombinant adenovirus onto the nares of deeply anesthetized mice. Control mice were immunized with replication-incompetent adenovirus expressing the LacZ gene (rAd-LacZ). Serum samples were collected 7 days after each immu-nization by retro-orbital bleeding and stored at -80°C until needed. Vaginal lavage fluid samples were obtained by infusing 100 μl of PBS (pH 7.2) into the vaginal canal and then recovering fluid with a micropipette. Vaginal lavages were collected

once per day for 3 days, and combined on day 7 post-immunization. Fecal samples were collected, weighed, and suspended at a concentration of 100 mg/ml in PBS containing 0.1% sodium azide. Each sample was stored at -80°C until use.

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

A standard ELISA was used to determine the levels of PrV-specific antibodies in the serum, vaginal lavage contents, and feces. Briefly, ELISA plates were coated overnight at 4°C with an opti-mal dilution (0.5 to 1.0 μg/well) of semi-purified PrV antigen to the sample wells, and either goat anti-mouse IgG (Southern Biotechnology Associate Inc., Birmingham, AL) or rabbit anti-mouse IgA (Zymed, San Francisco, CA) to 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) (Bianchi *et al.*, 1998). Next, 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 finally incubated with HRP-conjugated goat anti-mouse IgG for 1 h. Biotinylated goat-anti-mouse IgA was added to the samples for 2 h at 37°C to measure IgA levels in vaginal lavage fluid and in fecal samples, followed by the addition of peroxidase-conjugated strep-tavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). 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 pero-xide). The concentration of PrV-specific antibodies was determined using an automated ELISA reader and the SOFTmax Pro4.3 program and results were compared with two concentrations of stan-dard immunoglobulin proteins.

Th1- and Th2-type cytokine ELISA following *in vitro* stimulation of CD4$^+$ T cells

Mice were sacrificed two weeks after the final immu-nization for preparation of splenocytes and lymph node (LN) cells. Erythrocytes were depleted by the treatment of single cell suspensions with ammo-nium chloride containing Tris buffer (NH$_4$Cl-Tris) for 5 min at 37°C, and these cells were used as res-ponder cells. Enriched antigen-presenting cell (APC) populations were obtained using previously des-cribed methods (Eo *et al.*, 2001) and were used as stimulators. Briefly, splenocytes from naïve female

mice were depleted of erythrocytes, and 10^7 cells suspended in 3 ml of media were layered over 2 ml of a metrizamide gradient (Accurate Chemical and Sci., Westbury, NY; analytical grade with 14.5 g metrimazide added to 100 ml of PBS, pH 7.2). Cells were centrifuged at 600 × g for 10 min and the cell interface was collected. Next, enriched APC populations were pulsed with UV-inactivated PrV at 5.0 moi for 3 h (prior to inactivation), and cells were 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. Culture supernatants were harvested after 3 days of incubation, and a similar number of responder cells were stimulated for 48 h with 5 μg of concanavalin A as

a polyclonal positive stimulator.

Cytokine levels in the culture supernatants were measured with ELISA. Briefly, ELISA plates were coated with IL-2, IL-4, and IFN-γ anti-mouse antibodies (Pharmingen, San Diego, CA; clone no. JES6-1A12, 11B11, and R4-6A2, respectively) and incubated overnight at 4°C. The plates were washed three times with PBST and then blocked with 3% nonfat-dried milk for 2 h at 37°C. The culture supernatants and standards for recombinant IL-2, IL-4, and IFN-γ proteins (Pharmingen) were added to the plates, which were incubated for 2 h at 37°C. Biotinylated IL-2, IL-4, and IFN-γ antibodies (Pharmingen; clone no. JES6-5H4, BVD6-24G2, and XMG1.2, respectively) were then added and incubated overnight at 4°C. The plates were then
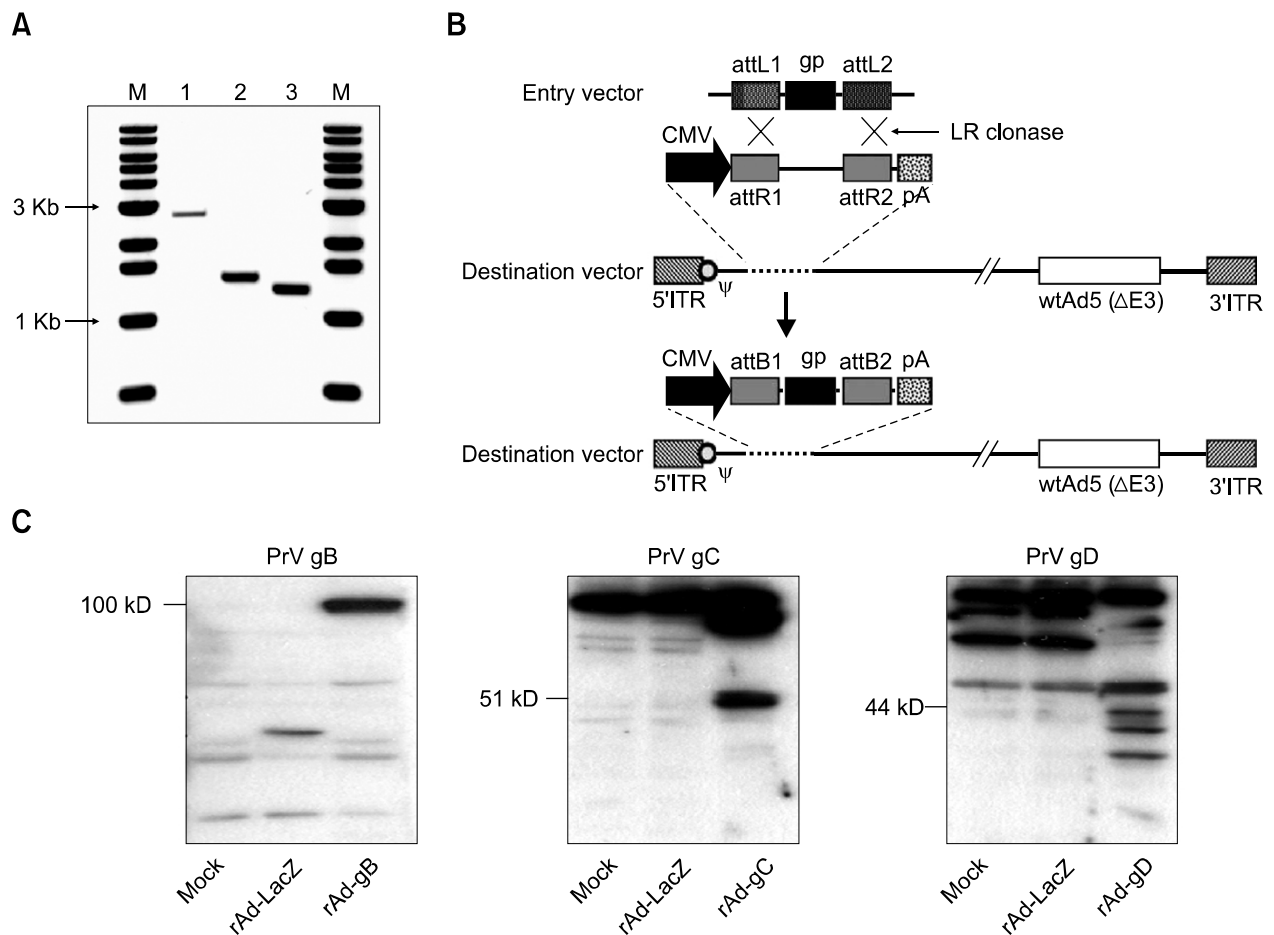
Figure 1. The rAd constructs used for this study. (A) PCR products of PrV glycoprotein genes (gB, gC, and gD). M, 1 kb DNA size marker ladder; 1, PrV gB gene (2901 bp); PrV gC gene (1615 bp); PrV gD gene (1323 bp). (B) Schematic representation of the linear genomes of the rAd expressing glycoproteins (gp) of PrV. The dashed line at the left end of the figure represents the position of region E1. The open box located toward the right end represents an approximately 2.7 kb deletion in the non-essential E3 region (△E3). The hatched boxes at either end represent the left and right inverted terminal repeats (5'ITR and 3'ITR, respectively), and the open circle near the 5'ITR represents the packaging signal (ψ). In the rAds, region E1 is replaced with the gp gene of the entry vector through recombination sites (attL1/R1, attL2/ attR2) in the presence of LR clonase. CMV, CMV promoter; pA, SV40 polyadenylation signal. (C) Identification of the expression of glycoprotein genes from rAds by immunoblot analysis. The protein extracts from NIH3T3 cells infected with purified replication-incompetent adenoviruses were analyzed by immunoblot analysis using polyclonal antibodies against gB, gC, and gD.

washed and incubated with peroxidase-conjugated streptavidin (Pharmingen) for 1 h, followed by color development. The cytokine concentration was determined using an automated ELISA reader.

Virus challenge experiment

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

Statistical analysis

Where specified, the data were analyzed for statistical significance using a Student's *t*-test. A *P* value < 0.05 was considered significant. Kaplan-Meier curves were generated for mice that survived a lethal challenge with PrV. The survival time of mice that were alive at the end of the study was regarded as censored. Time data were analyzed using the log rank statistic to compare the two survival curves, and *P* values were computed with the chi-square method. The survival rates of the two groups were considered to be significantly different if the two-side *P* value was less than 0.05.

Results

Construction of replication-incompetent adenovirus expressing glycoproteins

PCR products of each glycoprotein were amplified using specific primers, cloned, and sequenced to identify the ORF in order to construct replication-incompetent adenoviruses that express PrV gB, gC, and gD proteins (Figure 1A). Recombinant adenoviral expression plasmid DNA encoding glymbination of recombinant entry vectors in the presence of LR Clonase (Figure 1B). Following *Pac* I digestion and transfection into 293A cells, cells were observed to approximately 50-70% CPE. Recombinant adenoviruses expressing gB (rAd-gB), gC (rAd-gC), and gD (rAd-gD) were harvested and protein extracts from NIH3T3 cells infected with purified replication-incompetent adenoviruses were subjected to immunoblot analysis with polyclonal antibodies against gB, gC, and gD. The corresponding protein products of gB ($\sim$100 kD), gC ($\sim$51 kD), and gD ($\sim$44 kD) were detected in the reaction (Figure 1C), which indicated that replication-incompetent adenoviruses successfully expressed glycoproteins.

Comparison of immunity induced by replication-incompetent adenovirus expressing glycoproteins

In order to assess humoral responses induced by replication-incompetent adenoviruses expressing PrV glycoproteins, mice were immunized i.m. with rAd-gB, rAd-gC, and rAd-gD (10^6 pfu/mouse) through both tibialis muscles three times at 7 day intervals. PrV-specific IgG levels in the sera were quantified on the seventh day after each immunization, as described in Table 2. The control construct, replication-incompetent adenovirus expressing LacZ (rAd-LacZ), induced no significant PrV-specific IgG responses, while adenoviruses expressing each of the glycoproteins (rAd-gB, rAd-gC, and rAd-gD) produced detectable IgG

Table 2. Summary of serum PrV-specific IgG levels of the animals immunized with replication-defective adenovirus expressing glycoproteins, gB, gC, and gD.

Immunization[a]	Serum PrV-specific IgG (μg/ml)[b]			PrV-specific IgG isotypes (ng/ml)[c]		
	prime	boost 1	boost 2	IgG2a	IgG1	Ratio of IgG2a/IgG1
rAd-gB	0.52 ± 0.52	16.8 ± 5.81	105.9 ± 29.1[d,e]	80.7 ± 13.2[d,e]	26.3 ± 5.7	3.07
rAd-gC	0.34 ± 0.43	9.52 ± 5.60	56.3 ± 20.3	7.1 ± 8.9[f]	31.6 ± 19.4	0.22
rAd-gD	0.24 ± 0.26	7.27 ± 4.02	48.3 ± 19.1	18.9 ± 4.3	22.5 ± 2.3	0.84
rAd-LacZ	0.03 ± 0.01	0.42 ± 0.40	0.15 ± 0.16	-	-	-

[a]C57BL/6 (*n* = 7) mice were immunized i.m. with rAd expressing gB (rAd-gB), gC (rAd-gC), and gD (rAd-gD) as described in Materials and Methods. Control mice were given replication-incompetent adenovirus expressing LacZ gene (rAd-LacZ). [b]Seven days after each immunization, PrV-specific IgG levels in sera were determined by conventional ELISA. The values represent the average ± SD from seven mice per group. [c]Seven days after the final immunization, The levels of PrV-specific IgG isotypes (IgG2a and IgG1) in sera were determined by ELISA. The values represent the average ± SD from seven mice per group. [d]Significantly different from values obtained for rAd-gB-immunized group with rAd-gC-immunized group (*P* < 0.01). [e]Significantly different from values obtained for rAd-gB-immunized group with rAd-gD-immunized group (*P* < 0.01). [f]Significantly different from values obtained for rAd-gC-immunized group with rAd-gD-immunized group (*P* < 0.05).

levels after the first injection. Subsequent injections boosted the primary IgG responses. Replication-incompetent adenovirus expressing PrV gB (rAd-gB) induced the strongest responses of PrV-specific IgGs among the three constructs. In addition, both rAd-gC and rAd-gD revealed comarable levels of PrV-specific IgG in sera (Table 2). Evaluation of the PrV-specific IgG isotypes (IgG2a and IgG1) induced by each construct revealed that different isotype level patterns were observed in response to constructs expressing different immunogens (Table 2). The rAd-gB construct produced significantly higher amounts of the IgG2a isotype than rAd-gC and rAd-gD, resulting in the highest IgG2a to IgG1 ratio, which was indicative of a Th1-type immune response. Conversely, rAd-gC had a low IgG2a to IgG1 ratio as a result of higher production of the PrV-specific IgG1 isotype when compared to the

IgG2a isotype (Table 2). In addition, rAd-gD induced similar levels of IgG2a and IgG1 and produced a median IgG2a:IgG1 ratio. These results demonstrate that replication-incompetent adenoviruses expressing PrV glycoproteins successfully induced PrV-specific immunity. Further, different humoral responses were induced by adenoviruses that expressed different immunogens.

We examined the profile of Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines produced by CD4$^+$ T cells stimulated with the PrV antigen protein, which is known to induce the predominant expansion of immune CD4$^+$ T cells (Eo *et al.*, 2001). Replication-incompetent adenoviruses consistently induced a different pattern of Th1- and Th2-type cytokine production according to the expressed antigen (Figure 2). Animals immunized with rAd-gB displayed higher levels of IFN-γ production than those
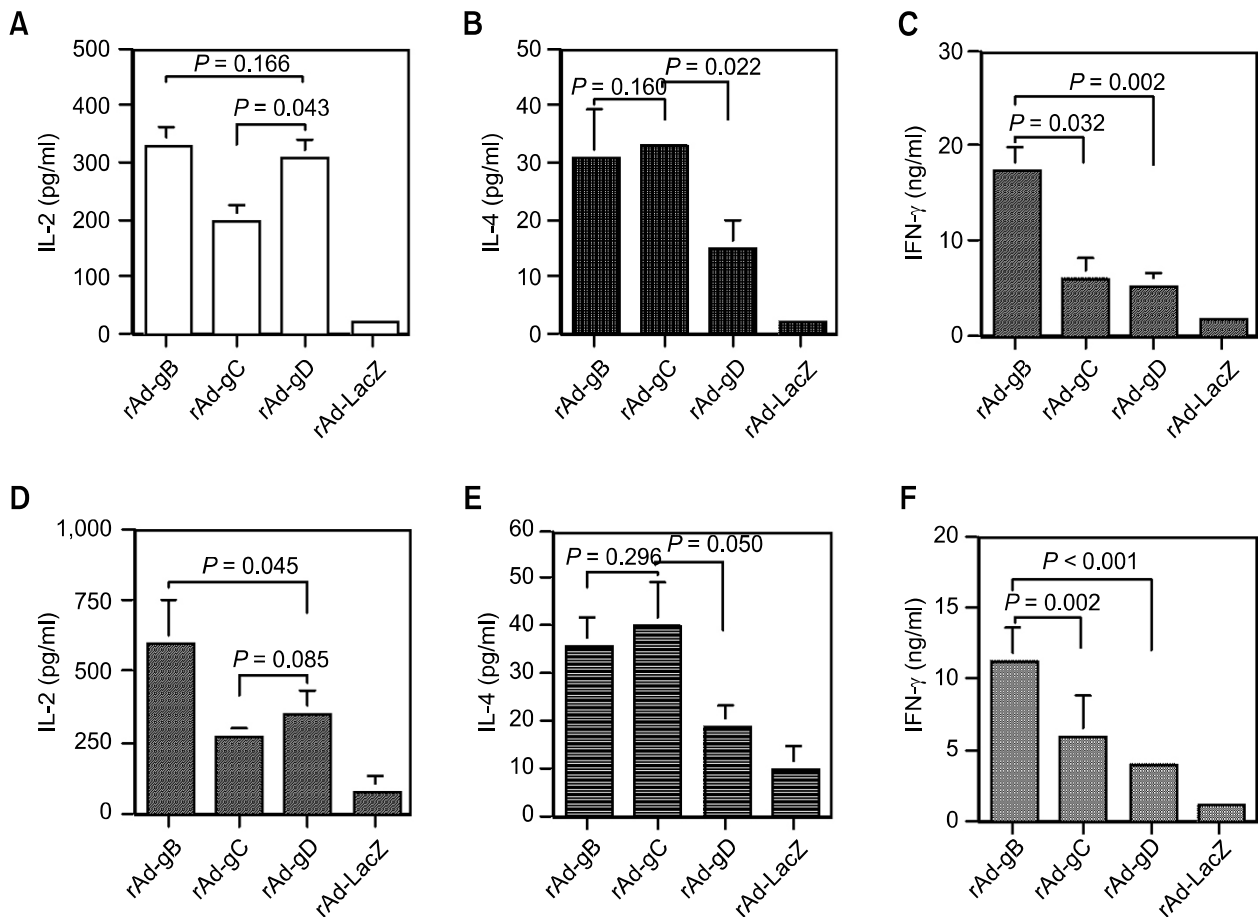
Figure 2. The profile of cytokine production (IL-2, IL-4, and IFN-γ) by splenocytes (A, B, and C) and popliteal LN cells (D, E, and F) from animals immunized with rAd expressing glycoproteins (rAd-gB, rAd-gC, and rAd-gD). The responder cells (splenocytes and LN cells from the immunized mice) were mixed with irradiated syngeneic enriched APCs that had been pulsed with UV-inactivated PrV, and incubated for 3 days at 2 weeks after the final immunization. The levels of cytokines in the supernatants of the stimulated T cells were determined by cytokine ELISA. The test was carried out in quadruplicate wells. The bars represent the average $\pm$ SD from three independent experiments. *P*-values in the graphs were calculated using the Student's *t*-test.

treated with rAd-gC and rAd-gD, and rAd-gC administration induced marginally higher IL-4 production than treatment with rAd-gB. Additionally, treatment with rAd-gD resulted in production of lower amounts of IFN-γ and IL-4, but not IL-2. These results and associated antibody responses indicated that rAd-gB induced Th1-type responses and rAd-gC induced Th2-type responses. In addition, rAd-gB was the most potent inducer of humoral and cellular immunity.

Immunity induced by systemic and mucosal delivery of replication-incompetent adenovirus expressing gB
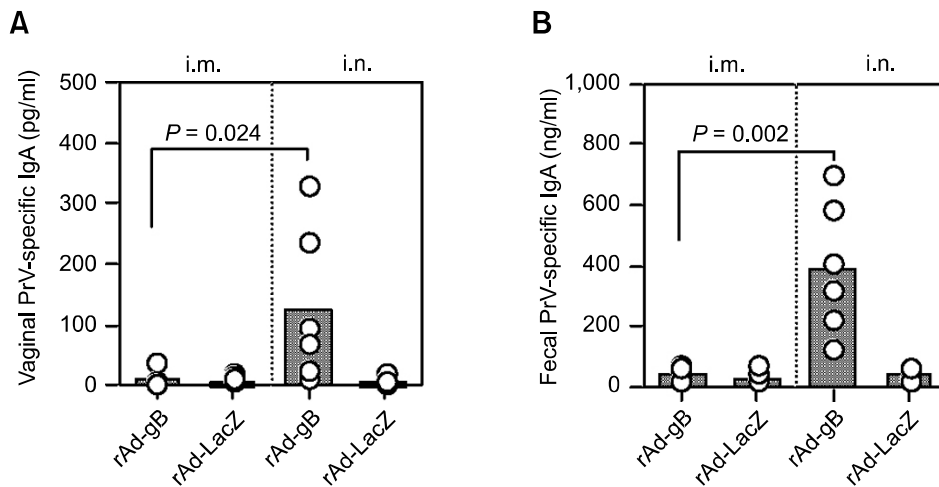
The immunity induced by systemic and mucosal delivery of replication-incompetent adenoviruses expressing gB (rAd-gB), which was the most potent immunogen among three constructs tested, was further evaluated. Groups of mice were immunized with rAd-gB by either the intramuscular

(systemic) or intranasal (mucosal) route. The i.n. immunization was performed by depositing rAd-gB onto the nares of deeply anesthetized mice. Detectable PrV-specific IgG antibodies in sera were produced by primary i.m. or i.n. administration of rAd-gB and boosted by subsequent immunizations (Figure 3A, B and C). However, the i.m. administration of rAd-gB showed stronger IgG responses than i.n. administration. In addition, when PrV-specific IgG isotype ratios were examined, i.n. administration induced a reduction in IgG2a to IgG1 ratios (Figure 3D).

Conversely, when IgA responses at mucosal sites were analyzed on the seventh day following the final immunization, induced IgA response levels displayed different patterns that were likely not a result of the serum IgG responses (Figure 4). Mice immunized i.m. with rAd-gB did not show significantly detectable PrV-specific IgA antibodies in the vaginal fluid or intestinal tract. However, i.n. administration of rAd-gB strongly induced secretory
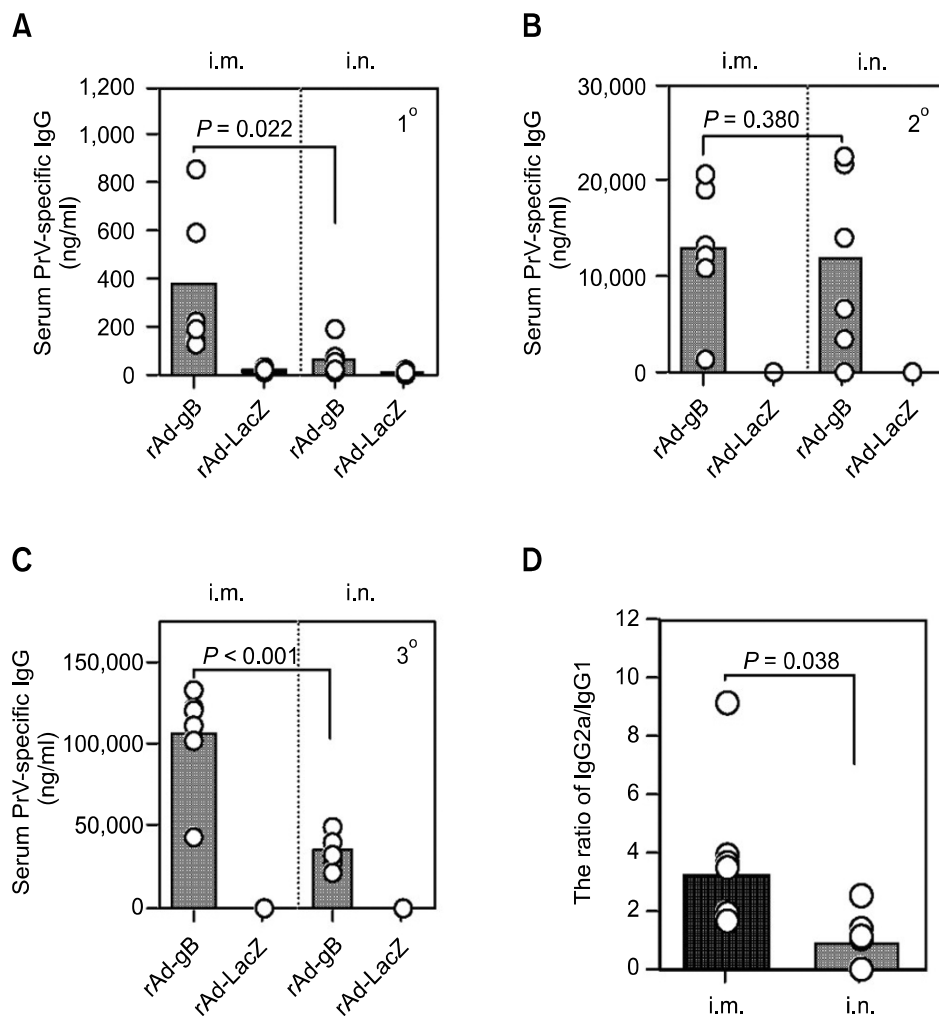
Figure 3. Serum PrV-specific IgG levels and associated isotype distributions in animals immunized by rAd-gB systemic and mucosal delivery. Groups of C57BL/6 (H-2b) mice were immunized with rAd-gB (10^6 pfu/mouse) by either the i.m. or i.n. route three times at 7 day intervals. On the seventh day after each immunization, the PrV-specific IgG levels in the sera were evaluated by ELISA. The levels of IgG2a and IgG1 were compared 7 days after the final immunization. The circles on the graph represent the individual IgG levels and the height of the bar shows the average of each group (n = 7). *P*-values in the graphs were calculated using the Student's *t*-test.

A

B

Figure 4. Mucosal IgA responses induced by systemic and mucosal delivery of rAd-gB. Groups of C57BL/6 (*n* = 7) mice were immunized with rAd-gB (10^6 pfu/mouse) by either the i.m. or i.n. route three times at 7 day intervals. Seven days after the final immunization, mucosal IgA levels in samples from both the vaginal tract (A) and feces (B) were measured by ELISA. The circles on the graph represent the individual IgG levels and the height of the bar shows the average of each group (*n* = 7). *P*-values in the graphs were calculated using the Student's *t*-test.

IgA responses at the mucosal site. These results suggest that mucosal IgA as well as serum IgG responses can be induced by mucosal delivery of rAd-gB. In addition, i.m. administration of rAd-gB produced strong serum PrV-specific IgG responses but not IgA responses at mucosal sites.

To further evaluate the nature of immunity induced by systemic and mucosal delivery of rAd-gB, the Th1 (IL-2 and IFN-γ)- and Th2 (IL-4)-type cytokines produced by CD4$^+$ T cells stimulated with antigen proteins were evaluated. Systemic and mucosal delivery of rAd-gB induced different patterns of Th1- and Th2-type cytokine production (Figure 5). Intramuscular administration of rAd-gB induced strong Th1-type cytokine (IFN-γ and IL-2) production. However, i.n. rAd-gB delivery revealed enhanced IL-4 production and reduced IFN-γ production. This pattern of Th1- and Th2-type cytokine production was consistent with those from PrV-specific IgG isotypes. Therefore, i.m. administration of rAd-gB induced Th1-type immunity and this was reduced by the i.n. delivery of rAd-gB.

Protective immunity induced by replication-incompetent adenovirus expressing glycoproteins against viral challenge

In order to compare the protective efficacy of immunity conferred by systemic and mucosal delivery of gB-expressing replication-incompetent adenovirus against a virulent viral infection, groups of mice that received i.m. and i.n. administration of rAd-gB were then challenged i.n. with the virulent PrV YS strain (10 LD$_{50}$) 2 weeks post-immunization. Anamnestic PrV-specific IgG and IgA in sera and vaginal lavages were evaluated 3 days after challenge, and serum IgG antibody levels in mice that received rAd-gB was comparable, regardless

of i.m. or i.n. administration (Figure 6A). Mice immunized i.m. with rAd-gB had significantly detectable IgA levels in the vaginal tract following challenge, although IgA levels in mice subjected to i.m. administration were marginally lower than in mice that received the i.n. administration (Figure 6B).

Survival curves of mice immunized with recombinant glycoprotein-expressing adenoviruses were examined. Both i.m. and i.n. delivery of rAd-gB provided effective resistance against viral challenge (*P* = 0.0002), as shown in Figure 7. In addition, the protective efficacy of rAd-gC administered via the i.m. route was inferior to that of the rAd-gB-immunized group (*P* = 0.0468), whereas the rAd-gD-immunized group showed marginally less protective immunity than the rAd-gB-immunized group (*P* = 0.1279). These results suggest that recombinant adenovirus expressing gB provides the most potent protection against a lethal challenge of virus. Further, rAd-gB delivered by both systemic and mucosal routes conferred comparable protective immunity.

Discussion

Overall, this study suggests that replication-incompetent adenoviruses expressing three major glycoproteins (gB, gC, and gD) of PrV induced both humoral and cell-mediated immune responses. Different patterns of immune responses emerged in response to adenoviruses that expressed different antigens. The recombinant adenovirus expressing gB (rAd-gB) elicited the most potent immune responses biased to Th1-type. Conversely, the adenovirus that expressed gC favored Th2-type responses, and the adenovirus that expressed

A

B

C

D

E

F
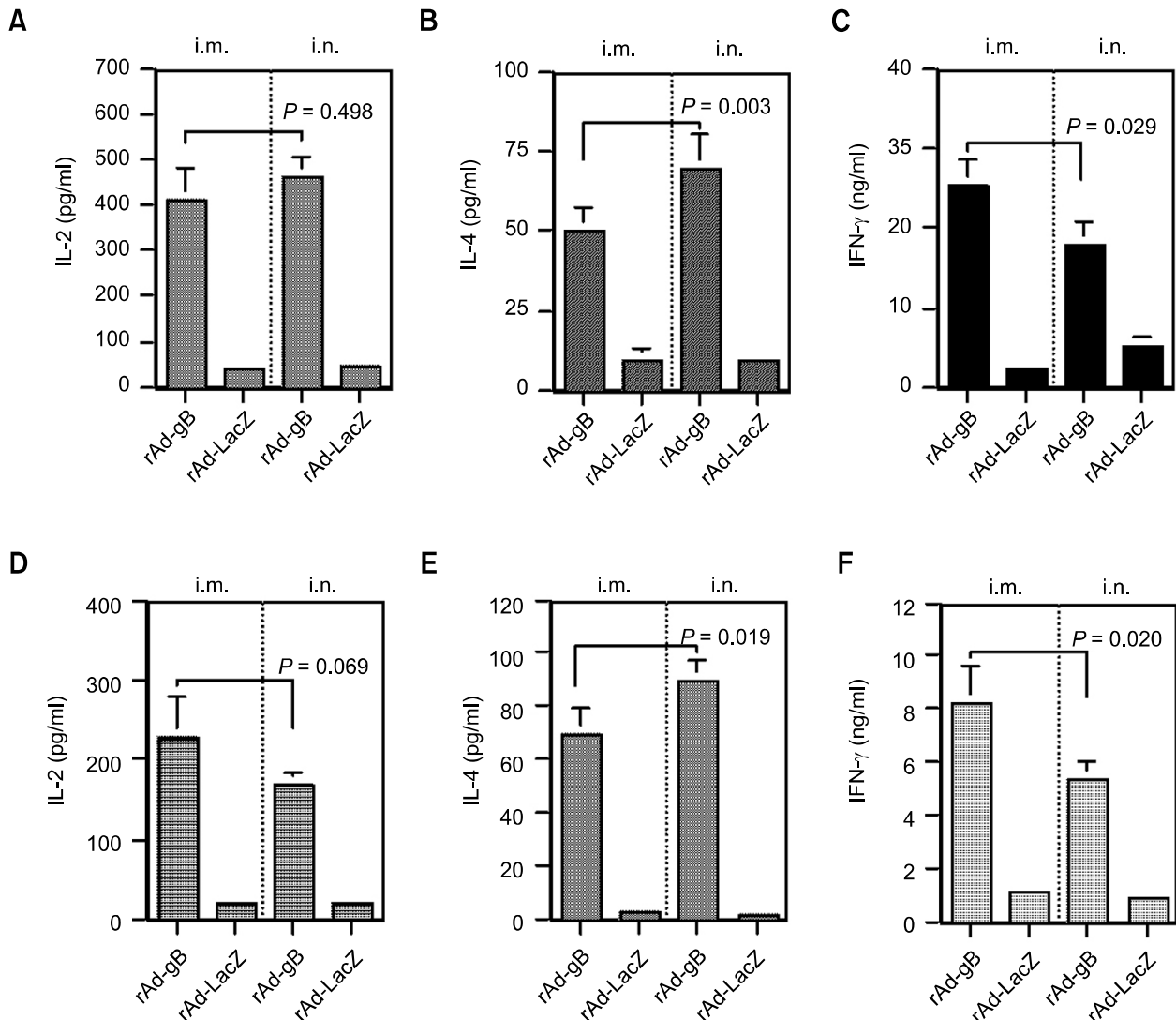
Figure 5. The profile of cytokine production (IL-2, IL-4, and IFN-γ) by splenocytes (A, B, and C) and draining LN cells (D, E, and F) of the animals immunized with rAd-gB via the systemic and mucosal routes. Responder cells (splenocytes and draining LN cells from the immunized mice) were mixed with the irradiated syngeneic enriched APCs, which had been pulsed with UV-inactivated PrV and then incubated for 3 days at two weeks after the final immunization. The levels of cytokines in the supernatants of the stimulated T cells were determined by cytokine ELISA. The test was carried out in quadruplicate wells. The bars represented the average ± SD of three independent experiments. *P*-values in the graphs were calculated using the Student's *t*-test.

rAd-gD induced lower IFN-γ and IL-4 levels than the other constructs, but revealed comparable IL-2 production. Mucosal delivery of rAd-gB induced mucosal IgA as well as serum IgG responses, and resulted in Th2-type immune responses. However, similar effects were not observed in response to systemic delivery. Finally, recombinant adenoviruses expressing gB conferred the most effective protection against a lethal viral challenge, regardless of systemic or mucosal administration.

A better knowledge of protective immunological parameters is a precondition in the development and effectiveness of anti-herpesvirus vaccines in general and against PrV in particular. There is increasing evidence that cell-mediated immunity is important in effective induction of protective immunity against herpesviruses (Bianchi *et al.*, 1998; Fischer *et al.*, 2000; Eo *et al.*, 2001; van Rooil *et al.*, 2004; La *et al.*, 2005), and the significance of Th1-type CD4^{+} effector T cells in protective immunity has been demonstrated with a PrV-infected murine model (Bianchi *et al.*, 1998; Yoon *et al.*, 2006). In our study, the replication-incompetent adenoviruses expressing gB showed Th1-biased immunity, which provided protection against PrV infection. This finding supports results from a

A

B
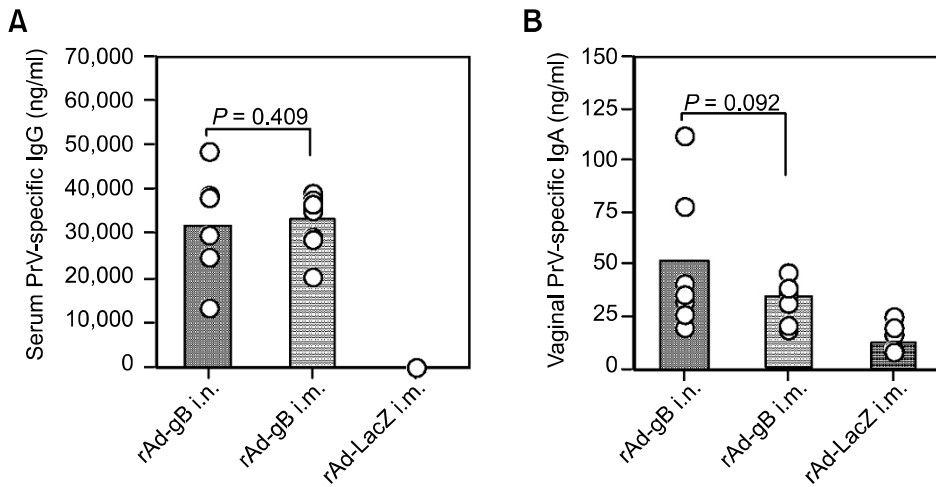
Figure 6. Serum PrV-specific IgG and mucosal IgA determined at 3 days post-challenge with the virulent PrV YS strain. Two weeks after the final immunization, groups of mice (*n* = 7) were challenged i.n. with the PrV YS strain (10 LD$_{50}$) and the sera and vaginal lavages were collected at 3 days post-challenge. PrV-specific IgG and IgA were determined by conventional ELISA. The circles on the graph represent the individual IgG levels and the height of the bar shows the average of each group. *P*-values in graphs were calculated using the Student's *t*-test.
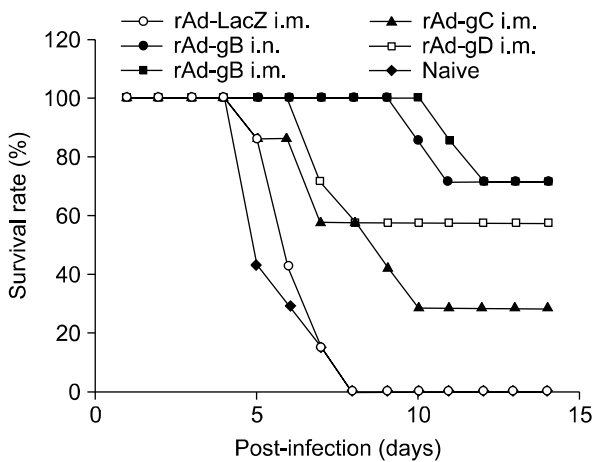
Figure 7. Susceptibility of animals immunized with rAd-gB via systemic and mucosal routes. Two weeks after the final immunization, groups of mice (*n* = 7) were challenged i.n. with the PrV YS strain (10LD$_{50}$). The challenged mice were then examined daily for any signs of inflammation, illness, and death until 14 days post-challenge. Kaplan-Meiers survival curves were computed and analyzed using the chi-square test..

previous report that illustrated the substantial role that gB plays as a dominant immunogen in the effective protection of a PrV DNA vaccination (Yoon *et al.*, 2006). In addition, the strongest responses of Th1-type CD4$^+$ T cells observed in this study were noted in mice that received gB-expressing plasmid DNA, which provided effective protection against a virulent viral infection (Yoon *et al.*, 2006). However, our results differed from another study which found that recombinant gD-expressing adenoviruses induced the most potent responses of neutralizing antibody of the three major glycoproteins (Monteil *et al.*, 1997), but with no absolute correlation between the presence of detectable neutralizing antibodies to gD prior to challenge and protection from disease. This su-

ggests that protection from PrV infection may be dependent on cell-mediated immunity. Therefore, our study results support previous studies that have demonstrated the important role that cell-mediated immunity, particularly Th1-type responses, plays in protective immunity against PrV infections.

Our study also suggested that different types of immunity were induced depending on the type of expressed antigen. Treatment with a replication-incompetent gB-expressing adenovirus biased to the Th1-type immunity and gC-expressing adenovirus induced a Th2-type responses. However, gD-expressing adenoviruses created confused immunity since rAd-gD induced production of the median ratio of IgG isotypes (IgG2a to IgG1) as well as production of IL-2 by stimulated CD4$^+$ T cells, which are one of the Th1-type cytokines. The nature of immunity induced by plasmid DNA expressing gD was biased towards Th2-type cells in a previous study of a PrV DNA vaccine (Yoon *et al.*, 2006). This difference in observation may result from properties of the vehicles expressing the antigens (plasmid DNA vs. adenovirus). A primary difference between transfers of plasmid- and adenovirus-mediated transgenes is that plasmid-mediated transgenes rely on the target cells expressing the foreign protein. The primary target cells for transfection upon i.m. inoculation of plasmid DNA are differentiated muscular cells. However, their role in stimulating a naïve immune system remains unclear because muscle cells express only low levels of MHC class I antigen and show no MHC class II antigen presentation (Shedlock *et al.*, 2000; Howarth *et al.*, 2004). It is therefore likely that antigen-presenting cells (APCs) are recruited into the plasmid DNA injection sites and initiate immune responses in draining LNs (Shedlock *et al.*, 2000; Howarth *et al.*, 2004), and even CpG

motifs in the backbone of plasmid DNA may affect the APC function in modulating immune responses to encoded antigens (Klinman *et al.*, 2006). Conversely, adenoviruses have a broad host cell range and can infect a wide spectrum of cells including quiescent or terminally differentiated cells (Davis *et al.*, 1993; Ali *et al.*, 1994; Randrianarison-Jewtoukoff *et al.*, 1995; Rocha *et al.*, 2004; Barouch *et al.*, 2005). Large numbers of small mononucleated cells were located within the endomysial spaces of muscle injected with adenovirus, and significant numbers of these cells expressed the reporter gene of the recombinant adenovirus (Davis *et al.*, 1993). These mononucleated cells are not present in normal or regenerating muscle injected with plasmid DNA and were identified as blood cells (Davis *et al.*, 1993). These results suggest that there are important differences in the initiation of immune responses between plasmid- and adenovirus-mediated vaccines.

The nasal mucosa is an important arm of the mucosal immune system and is generally the first entry site of several inhaled pathogens including PrV (Davis, 2001). Therefore, the nasal mucosa represents an attractive, noninvasive delivery route for several vaccines. A particular advantage of i.n. vaccine delivery is the requirement for a smaller quantity of antigen by this route when compared to the amount needed for oral immunization (Davis, 2001). Results from our study suggest that the i.n. delivery of replication-incompetent gB-expressing adenoviruses engaged the common mucosal immune system and induced significant immunity at both the systemic and distal mucosal sites, but that i.m. injection produced no IgA at the mucosal site. Further, mucosal delivery of rAd-gB induced Th2-type immunity when compared to systemic delivery. This suggests that the delivery route of vaccines may partially determine the nature of induced immune responses to antigens, as supported by previous studies (McCluskie *et al.*, 1999; Okamba *et al.*, 2007; Yoon *et al.*, 2008). Both systemic and mucosal delivery of rAd-gB provided comparable protection. Curiously, the i.m. delivery of rAd-gB elicited detectable IgA production similar to that in groups that received treatment by the i.n. route 3 days after PrV challenge (Figure 6B). However, as was documented previously, it appears that immunity to PrV may depend more on a functioning T-cell defense system in infected tissues rather than on mucosal IgA function in the mucosal tissues (Bianchi *et al.*, 1998; van Rooij *et al.*, 2000). This is supported by a study that found similar protective immunity against human herpesvirus in both IgA knockout and wild-type mice (Parr *et al.*, 1998). Therefore, both the cell-mediated and

humoral immune responses systemically induced by rAd-gB may play critical roles in protection against PrV infection.

Adenovirus vectors are increasingly used for a variety of *in vivo* gene transfer experiments including vaccination. Replication-incompetent recombinants do not disseminate further because they undergo no additional rounds of replication, and thereby this replication-incompetent adenovirus vector is biologically safe (Babiuk *et al.*, 2000). Recombinant adenovirus vaccines are particularly useful in the vaccination of neonates immediately after birth independent of the presence or absence of maternal antibodies (Monteil *et al.*, 1997, 2000; Wesley *et al.*, 2004; Liu *et al.*, 2007). Therefore, there is significant veterinary utility for recombinant adenovirus vaccination of pigs slaughtered within a few months of birth and of pigs as young as 1 day of age, as such vaccines will not encounter maternal antibody interference. The data obtained from the PrV infection using murine models may reflect the same immunological parameters that allow vaccine for protection of pigs, since the importance of Th1-type $CD4^+$ T cells and their IFN-γ production have been observed in both species (Bianchi *et al.*, 1998; Ficher et al., 2000; Eo *et al.*, 2001; van RooiJ *et al.*, 2004). In conclusion, the results of this study suggest that replication-incompetent gB-expressing adenovirus induces Th1-biased humoral and cellular immune responses and provides effective protection against virulent virus infection. Further development of vaccine protocols using recombinant adenoviruses and DNA vaccines will sharpen their usefulness in the protection against pathogens such as PrV.

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