DNA PRIMING AND KILLED VIRUS BOOSTING VACCINATION STRATEGY CAN AUGMENT THE IMMUNE RESPONSE TO PSEUDORABIES VIRUS

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Summary. – The immune efficacy of DNA vaccines containing three plasmids encoding gB, gC, and gD glycoproteins (Mix DNA) of Pseudorabies virus (PRV) or the plasmid for gC only (gC DNA), killed virus (KV) vaccine or combination of gC DNA, Mix DNA and KV vaccines was evaluated in mice using prime-boost strategy. The mice vaccinated twice with Mix DNA, and once with KV generated higher levels of gC-specific and virus neutralization (VN) antibodies and a stronger cellular immune response than the mice vaccinated three times with the Mix DNA vaccine only. The highest level of VN antibodies were detected in mice vaccinated twice with KV vaccines alone or with combination of DNA and KV vaccines. The challenge of vaccinated mice with the lethal dose of PRV showed that the complete protection against PRV was achieved in the group of mice immunized with the DNA and KV vaccines combined. The results suggested that DNA priming followed by KV vaccine boosting could enhance the antibody response and cellular immunity against PRV infection in mice.

Key words: Pseudorabies virus; glycoprotein; DNA vaccine; killed virus vaccine; prime-boost strategy; immune response

Introduction

Aujeszky's disease (AD) caused by PRV is a serious infectious disease in pig farming. An active immunization with the modified live or KV vaccines is widely carried out to control this disease (Mengeling *et al*., 1997). Although these vaccines are highly effective against clinical signs of AD, they do not completely prevent infection, excretion and establishment of latency of PRV (Mulder *et al*., 1995). The KV vaccine widely used in China could elicit VN antibodies and was safer than PRV gene-deleted vaccines and other live virus-vector vaccines (Chen *et al*., 2001). However, the immune protection mediated via cellular immunity is lacking that is considered as an essential part of immune efficiency against PRV infection (van Rooij *et al*., 2004).

Different approaches to develop an effective vaccine against PRV were explored as the killed whole-virus vaccine with cytokine adjuvant, live vector vaccine, and DNA vaccines (Gerdts *et al*., 1999; Fischer *et al*., 2003; Lin *et al*., 2005). Previous studies showed that DNA vaccine expressing major envelope glycoprotein gB, gC, and gD of PRV could provide protective immunity in pigs. Vaccination with DNA plasmids encoding PRV glycoprotein gC or gD required three successive injections and elicited only partial protection in pigs (Gerdts *et al*., 1997). Different strategies have been proposed as a possible means of DNA vaccines improvement (Somasundaram *et al*., 1999; van Rooij *et al*., 2002). Thus,

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Abbreviations: AD = Aujezsky's disease; gC DNA = pcDNA3.1-gC; Mix DNA = mixture of pcDNA3.1-gB, -gC, -gD; KV = killed virus; PRV = Pseudorabies virus; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LTT = lymphocyte transformation test; DTH = delayed-type hypersensitivity; VN = virus neutralization

the development of a new vaccination strategy is essential for improving immune efficiency of DNA vaccination. In recent years, prime-boost regimen was considered as the worthy means for enhancement of an immune response in various diseases (McShane, 2002; Doria-Rose and Haigwood, 2003). The priming with plasmid DNA followed by the boosting with the different type of vaccine was shown to synergistically induce a higher immune response than separate administration of individual vaccines (Sin *et al*., 1999; Toussaint *et al*., 2005).

In this report, we described the DNA priming and KV vaccine boosting approach in an attempt to improve the immune response in vaccinated mice by comparing the immune efficacy of DNA priming with gC DNA or with a cocktail of plasmids encoding gB, gC, and gD glycoproteins in combination with KV vaccine. Our results showed that DNA priming followed by KV vaccine boosting could enhance the antibody response and cellular immunity against PRV infection in mice.

Materials and Methods

Cells and virus. BHK-21 cells were cultivated in DMEM (Gibco) supplemented with 10% of inactivated fetal bovine serum (FBS, Gibco), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C in 5% CO_2. The PRV strain Fa (kindly provided by Fujian Academy of Agricultural Science, China) was propagated in BHK-21 cells. LD_{50} of PRV was determined in mice (Yin and Liu, 1997). PRV with a titer of 2×10^7 $TCID_{50}$/ml was inactivated by radial γ for 24 hrs and used in experiments calling for the inactivated virus.

Preparation of the recombinant gC protein. To obtain the recombinant gC protein *in vitro*, N-terminal domain of gC protein (32–302aa) (Ober *et al*., 2000) was expressed in the IMPACTTM-pMXB10 system (New England Biolabs Inc.). After SDS-PAGE, the expressed recombinant protein was eluted from the gel in the dialysis tubing and stored at -80°C.

Eukaryotic plasmid constructs for immunization. Viral DNA was prepared from BHK-21 cells infected with PRV strain Fa and used as a template for PCR. Primers (gB-F1/R1, gC-F1/R1, gD-F1/R1) for amplification of gB, gC, and gD gene were designed according to the complete genomic sequence of PRV (Klupp *et al*., 2004) (Table 1). The *Eco*RI and *Xba*I restriction sites were introduced into upstream and downstream primers at 5'-end. The full-length fragments of gB, gC, and gD gene amplified by PCR were inserted into the *Eco*RI and *Xba*I sites of the eukaryotic expression vector pcDNA3.1 (Invitrogen) and their insertion was confirmed by sequencing. The recombinant DNA expressing gB, gC, and gD gene was transformed into *Escherichia coli* DH5α, extracted, and purified by PEG 8000 precipitation (Sambrook and Russell, 2001). The final products were dissolved in PBS and stored at 4°C until used.

Immunofluorescence assay. BHK-21 cells were seeded at 4×10^5 cells to a 6 cm^2 plate containing DMEM, supplemented with 5% FBS and cultivated to 80% confluency. The BHK-21 cells were transfected with plasmids pcDNA3.1-gB, gC, gD, and control pcDNA3.1 using the Lipofectamine 2000 (Invitrogen). After 48 hrs, the transfected cells were trypsinized and seeded into 96-well plate. After 5 hrs of incubation, the cells were washed twice with PBS and fixed with ice-cold methanol for 5 mins. The fixed cells were incubated with mouse anti-PRV serum diluted 1:20 in PBS

Table 1. Oligonucleotide primers used for PCR

Primer	Sequence (5'-3')[a]	PRV genome stretch
gB-F1	TAT GAATTCGCC ACCATGCCCGCTGGTCTTTGGCG	19575–19595
gB-R1	CAGTCT AGATTACAGGGCGTCGGGGTCCTCGCTCTCGAG	16884–16914
gC-F1	ATAGAATTCGCCACCATGGCCTCGCTCGCGCGTGCGATG	54029–54053
gC-R1	AGCTCTAGATTACAGCACGGGCCGGCGATAGTAGACG	55440–55468
gD-F1	TTAGAATTCGCCACCATGCTGCTCGCAGCGCTATTGGCGG	121075–121100
gD-R1	ACCTCTAGATTACGGACCGGGCTGCGCTTTTAGCTC	122251–122277

[a]*Eco*RI and *Xba*I restriction sites are underlined.

Table 2. Immunization schemes of BALB/c mice

Group	Prime	Boost	Challenge
gC DNA/KV	gC DNA 0wk, 2wk	KV 4wk	7wk
gC DNA/gC DNA	gC DNA 0wk, 2wk	gC DNA 4wk	7wk
Mix DNA/KV	Mix DNA 0wk, 2wk	KV 4wk	7wk
Mix DNA/Mix DNA	Mix DNA 0wk, 2wk	Mix DNA 4wk	7wk
KV/KV	KV 0wk	KV 3wk	6wk
pcDNA3.1 control	pcDNA3.1 0wk, 2wk	pcDNA3.1 4wk	7wk
PBS control	PBS 0wk, 2wk	PBS 4wk	7wk

with 0.05% Tween-20 and 5% goat serum. After washing, the cells were incubated with FITC-goat anti-mouse IgG (Santa Cruz Biotechnology) for 30 mins, washed and visualized under fluorescent microscope.

Immunizations of mice. Female BALB/c mice (6–8-week-old), 18 mice in the group, were immunized according to the schedule shown in Table 2. Each DNA plasmid (100 μg/dose for gC; 50 μg/dose for gB and gD) was prepared in volume of 100 μl of PBS and administered intramuscularly 3 times into separate sites of the quadriceps muscle. The formalin-killed PRV vaccine in oil emulsion was administered subcutaneously and intramuscularly in the volume of 100 μl/dose (Chen *et al*., 2001). The group of mice receiving the KV vaccine only was immunized 2 times at 3-week interval. The immunized animals were bled by the tail vein puncture.

ELISA. The 96-well ELISA plates were coated overnight at 4°C with 200 ng/100 μl of the recombinant gC protein in 50 mmol/l sodium carbonate buffer and blocked for 1 hr with blocking buffer (1% gelatin in PBST). Serum samples diluted in blocking buffer in the volume 100 μl/well were added in duplicate and incubated for 1 hr at 37°C. Then, 100 μl of HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) diluted 1:6,000 in blocking buffer was added to each well and the plates were incubated at 37°C for 1 hr. 50 μl of substrate solution 3,3'-5,5'-tetramethyl benzidine was added to each well for 8 min and the reaction was stopped by addition of 50 μl of 1% H_2SO_4. The A_{450} was read in the ELISA reader. Antibody titers were determined as the highest serum dilution yielding ≥2.1 ratio of tested serum and control serum (A_{450}).

Virus neutralization assay (VN) was carried out by mixing equal volumes of 200 $TCID_{50}$ of PRV Fa strain with two-fold dilutions of tested serum in 96-well plates in triplicates. After incubation at 37°C for 24 hrs, the suspension of BHK-21 cells containing 1×10^4 cells in 100 μl was added to each well and plates were incubated for 5 days. The VN titer was expressed as $\log_2$ of reciprocal of the highest serum dilution showing the complete inhibition of CPE.

Lymphocyte transformation test (LTT). The spleen was excised from immunized mice (N = 4) and homogenized in PBS. The erythrocytes present in cell suspension were lysed with 0.75% Tris-NH_4Cl (pH 7.4) and single-cell suspension of splenocytes (2×10^5/100 μl) was plated in 96-well plates. The cells were stimulated with 10 μl of radial γ-inactivated PRV. 5 μg/ml of concanavalin A (Sigma) was used as the positive control and non-stimulated cell as the negative control. All assays were performed in triplicates. The proliferative response was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay (Li *et al*., 2006). Briefly, after stimulation with PRV or concanavalin A for 72 hrs, 20 μl of MTT was added to each well and plates were incubated for 4 hrs. The A_{570} value was read with the plate reader. The stimulation index (SI) was determined by the ratio of mean A_{570} reading of triplicate antigen-containing wells to mean A_{570} reading of triplicate wells without antigen.

Delayed-type hypersensitivity (DTH) test. DTH response to PRV was tested on day 21 post immunization in each immunization group containing four mice. PRV strain Fa with a titer of 10^6 $TCID_{50}$/ml was injected in 50 μl volumes in the right footpad as the test antigen. BHK-21 cell extract was injected in the same volume in the right footpad as a negative control. The footpad thickness was measured with an engineer's micrometer at 0, 24 and 48 hrs after the injection. The DTH reaction was recorded by calculating the value (increase of left footpad thickness after injection with PRV – increase of right footpad thickness after injection with BHK-21 extract). Data were expressed as means ± SD (N = 4).

Challenge of vaccinated mice with PRV. Ten mice in each vaccination group were challenged with 20 LD_{50} of PRV strain Fa (approximately 10^5 $TCID_{50}$) by the intrafootpad and intraperitoneal injection at day 21 after the last immunization. The challenged animals were observed daily for 15 days for survival and clinical signs such as herpetic lesions and paralysis.

Statistical analysis. Student's *t*-test was used for comparison between arithmetical means of humoral and cellular immune responses. *P* values of <0.05 were considered as statistically significant.

Results

Expression of DNA plasmids in BHK-21 cells

The full-length coding fragments of 915 aa for gB, 488 aa for gC, and 405 aa for gD were amplified and cloned into expression vector pcDNA3.1. The plasmids were designated as pcDNA3.1-gB, pcDNA3.1-gC, and pcDNA3.1-gD, respectively. Immunofluorescence assay showed that BHK-21 cells transfected with pcDNA3.1-gB, pcDNA3.1-gC and pcDNA3.1-gD could exhibit specific immunofluorescence indicating that the DNA plasmids encoding gB, gC, and gD gene of PRV could be successfully expressed in eukaryotic cells (Fig. 1A, B, and C). The cells transfected with control plasmid pcDNA3.1 showed only background fluorescence (Fig. 1D).

Antibody response to gC

Antibodies specific to gC protein of PRV were tested in ELISA with recombinant gC protein as an antigen. The gC-specific antibodies were detected in sera of mice immunized with gC DNA, Mix DNA plasmids or KV vaccine alone or in combination (Table 2). After the third injection the level of specific gC-specific antibodies was much higher than after first injection ($P < 0.05$) in all vaccinated groups (Fig. 2). After the third injection, the titers of gC-specific antibodies in the group of mice that received gC DNA or Mix DNA prime followed by the KV vaccine boosting were significantly higher than in the groups of mice receiving gC DNA or Mix DNA vaccines alone. Among all vaccinations, the group of mice receiving gC DNA double prime followed by KV boost induced the highest titers of gC-specific antibodies. Moreover, the titers of gC-specific

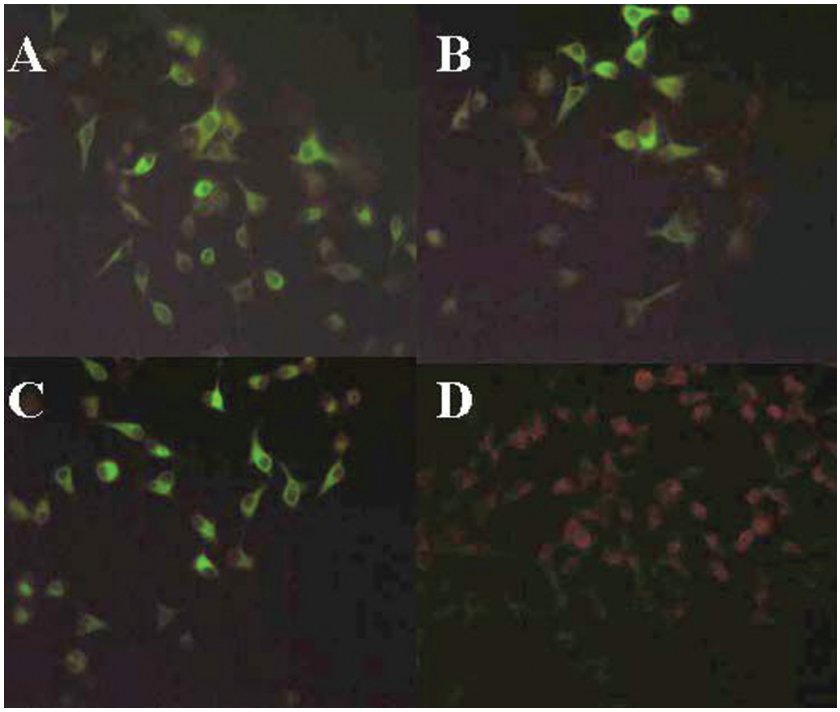

Fig. 1

Expression of gB, gC, and gD of PRV in BHK-21 cells detected by immunofluorescence assay

pcDNA3.1-gB (A), pcDNA3.1-gC (B), pcDNA3.1-gD (C), and control pcDNA3.1 (D).

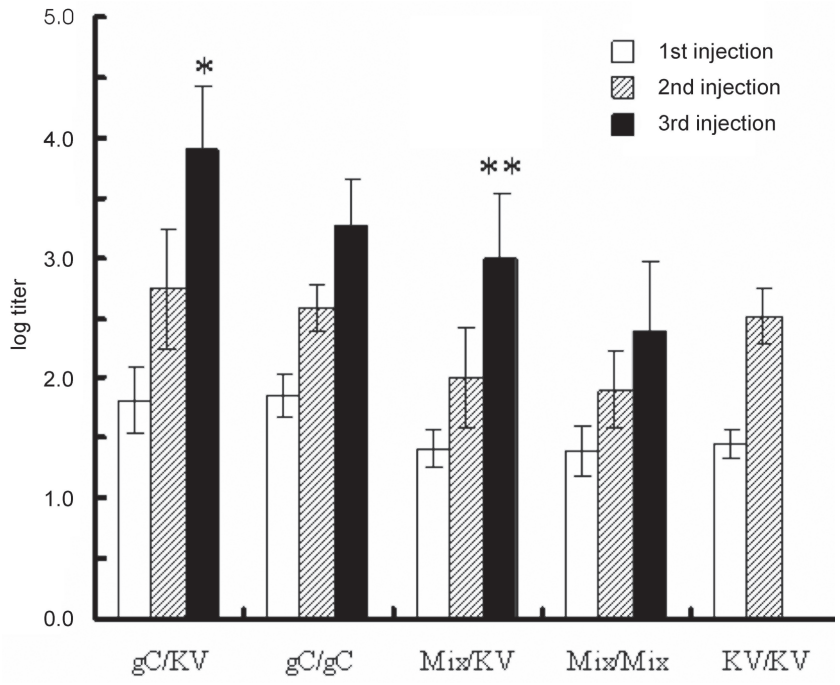

Fig. 2

ELISA titers of gC-specific antibodies in groups of mice vaccinated by gC DNA, Mix DNA or KV vaccine alone or combined

*Statistically significant value ($P < 0.05$) compared with the KV/KV or gC DNA/gC DNA vaccination groups. **Statistically significant value compared with the Mix DNA/Mix DNA vaccination groups.

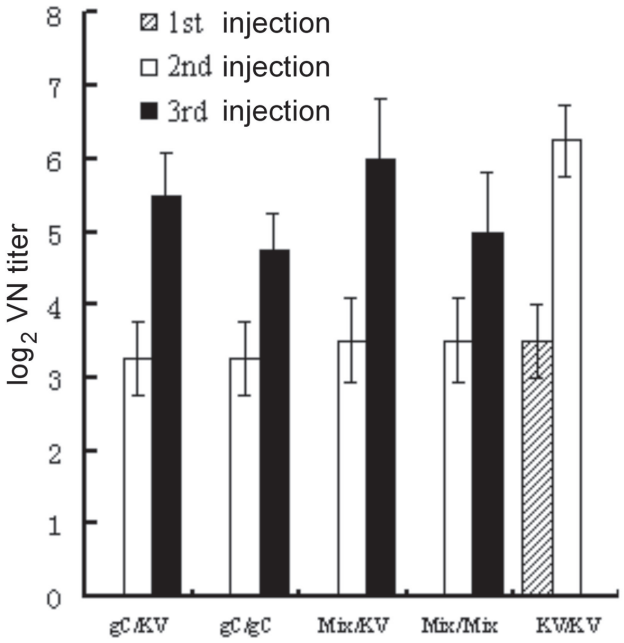

Fig. 3

VN titers in mice immunized with gC DNA, Mix DNA or KV vaccine alone or combined

The VN assays were performed after 2nd and 3rd immunization, with the exception of group immunized with KV vaccine only and tested after 1st and 2nd immunization.

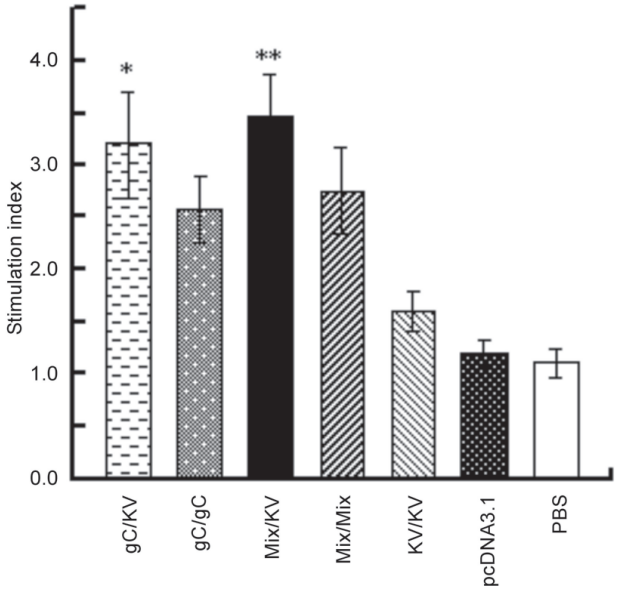

Fig. 4

LTT response in mice vaccinated with gC DNA, Mix DNA, and KV vaccines alone or combined

*Indicated values were statistically significant ($P < 0.05$) compared with KV/KV or gC DNA/gC DNA groups. **Indicated values were statistically significant compared with KV/KV or Mix DNA/Mix DNA groups.

antibodies in the group of Mix DNA alone or with a boost of KV were always lower than those in the group of gC DNA alone or with a boost of KV. Immunization of mice with control pcDNA3.1 or PBS showed no detectable gC-specific antibodies (data not shown).

Detection of VN antibodies

Before the last injection, the titers of VN antibodies in all vaccination groups were similar to each other ($\log_2$ titer = 3.0 – 3.6), regardless the mice were immunized with individual or combined vaccines (Fig. 3). After the last inoculation, the highest level of VN antibodies was observed in the sera of mice immunized with KV vaccine only. However, the mean titers of VN antibodies in the sera of mice vaccinated with gC DNA/KV or Mix DNA/KV vaccines were higher than that of mice vaccinated with gC DNA or Mix DNA plasmids only. The titer of VN antibodies in the group of mice vaccinated with Mix DNA/KV combination was similar to the VN titer in the group vaccinated with KV/KV ($P > 0.05$). The titer of VN antibodies in the group of Mix DNA/Mix DNA or Mix DNA/KV was higher than that in the group of gC DNA/gC DNA or gC DNA/KV.

Splenocyte transformation induced by gC DNA, Mix DNA and KV vaccines only or combined

Overall, the SI values detected in the splenocytes of mice immunized with gC DNA or Mix DNA only or in combination with KV vaccine was significantly higher than those of mice immunized with KV vaccine alone or with the control pcDNA3.1 in LTT ($P < 0.05$). The highest SI = 3.37 was detected in the splenocytes of mice vaccinated with Mix DNA followed by KV boosting (Fig. 4). The combined vaccinations with gC DNA/KV vaccine or Mix DNA/KV vaccine showed higher level of LTT response than vaccination with gC DNA or Mix DNA only.

PRV-dependent DTH responses

On day 21 after the last immunization, the PRV-specific DTH response was tested. The group of mice vaccinated with the Mix DNA/KV showed the highest level of DTH response, followed by the group of mice immunized with the gC DNA/KV vaccines combined (Fig. 5). A weaker DTH reaction was observed in groups of mice immunized with gC DNA or Mix DNA vaccines only. Apparently, the extent of DTH response in those groups of mice was significantly

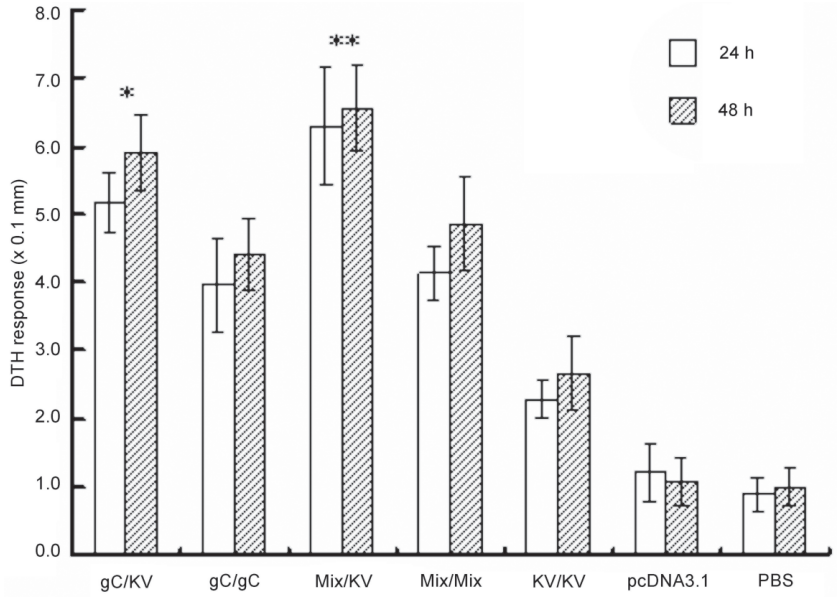

Fig. 5

DTH response in mice vaccinated with gC DNA, Mix DNA, and KV vaccines alone or combined

*Indicated values were statistically significant (P <0.05) compared with the KV/KV or gC DNA/gC DNA groups. **Indicated values were statistically significant in comparison with the KV/KV or Mix DNA/Mix DNA groups.

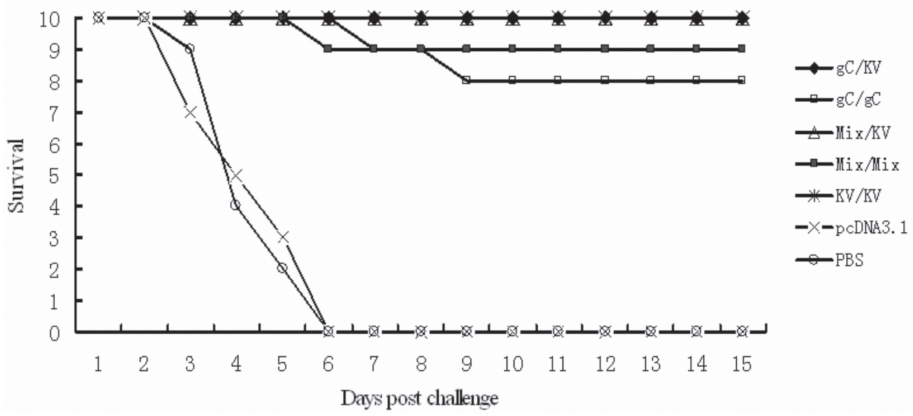

Fig. 6

Protection of the vaccinated mice against lethal PRV challenge

increased by the KV boosting (P <0.01). A weak DTH reaction was detected in mice vaccinated with KV vaccine only or with control pcDNA3.1.

Protection of mice against lethal challenge of PRV

Mice were vaccinated with gC DNA, Mix DNA or KV vaccine individually or combined and then lethally challenged with PRV by the intra-footpad and intraperitoneal injection**.** The groups of mice vaccinated with KV vaccine only or with the vaccination plan gC DNA/KV and Mix DNA/KV showed a survival rate of 100% (10/10) as compared with the group of mice vaccinated with control pcDNA3.1 or with PBS (P <0.01). The mice immunized with gC DNA or Mix DNA vaccines only showed a survival rate of 80% (8/10) or 90% (9/10), respectively (Fig. 6). All mice vaccinated with the control pcDNA3.1 or PBS died by day 7 after challenge.

Discussion

In this paper, we evaluated the protection efficacy of three different immunization strategies (DNA/DNA, KV/KV or DNA/KV) for PRV vaccines in BALB/c mice. The combined vaccination DNA/KV groups displayed significantly higher levels of humoral response (titer of gC-specific antibodies and VN titer) and the extent of cellular immunity (LTT and DTH response) when compared to DNA/DNA vaccination groups. The highest level of VN antibodies and the complete protection against lethal challenge of PRV were found in KV/KV groups, but the level of cellular immunity was weak. In addition, the VN antibody titer of Mix DNA/KV group was very similar to that of KV/KV group. Our data demonstrated that the immune response elicited by three vaccination plans were effective in controlling the PRV challenge, although the DNA/DNA vaccinated group had significantly lower protection efficiency (80%–90%) as compared to KV/KV (100%) and DNA/KV (100%) groups.

Previous data indicated the essential role of the CD4+ cells for protective immunity against lethal challenge with PRV in the mouse model (Dufour and Boisseson, 2003). The optimal protection was associated with a quick recall of the helper T-cell response, protection against clinical signs and a strong reduction of virus excretion in pigs (Bianchi *et al*., 1998). Our data related to LTT and DTH response suggested that the optimal vaccination could be performed with the combination of DNA and KV vaccines. In our study, gC DNA or Mix DNA plasmids only induced a lower level of gC-specific and VN antibodies as compared to KV vaccine only. This discrepancy might be due to the intracellular presence of the recombinant polypeptide expressed by the plasmid that was not fully available for processing of antigen presenting cells.

gC DNA induced higher level of gC-specific antibodies than Mix DNA vaccine, lower level of VN antibodies and also lower cellular response, suggesting that Mix DNA vaccine containing multiple genes possessed stronger capacity to activate immune system than the single gene. The mice receiving the Mix DNA inoculations followed by the KV boost had the highest LTT and DTH response, and higher titer of VN antibodies to PRV. The result indicated that immunization effects of multiple antigen DNA in a formulation, used in a protocol of DNA prime-protein boost, could be further improved compared to the individual DNA.

Two doses of the conventional vaccine were nearly as effective as our combined DNA/KV regime, suggesting a DNA/KV regime may still offer certain advantages. The DNA/KV regime was ultimately proved to be more protective than two doses of conventional vaccine, since the cellular immunity assayed in the DNA/KV cohort versus KV/KV cohort were clearly stronger. However, this difference needs to be confirmed in experiments with much larger numbers of animals on virus excretion and latency or resistance against higher lethal dose virus attack. In addition, plasmid vaccine was not affected by maternally derived immunity, while immunization with the inactivated vaccine might be impeded by passive immunity. Therefore, DNA/KV regime could provide more resistance against maternal interference.

In conclusion, our results provide valuable information regarding the prime/boost vaccination strategy against PRV to overcome the possible deficiencies of an individual vaccine regimen. We assume that this issue will be very important for PRV vaccine development in the near future.

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