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RESEARCH ARTICLE

Construction and immunogenicity of recombinant pseudorabies virus expressing the modified GP5m protein of porcine reproduction and respiratory syndrome virus

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Abstract Pseudorabies virus (PRV), an alpha-herpesvirus, has been developed as a live viral vector for animal vaccines. However, the PRV recombinant virus $TK^-/gE^-/GP5^+$ expressing GP5 of porcine reproductive and respiratory syndrome virus (PRRSV), based on the PRV genetically depleted vaccine strain $TK^-/gE^-/LacZ^+$, scarcely stimulated the vaccinated animals to produce neutralizing antibodies against PRRSV. To develop a booster-specific immune response of such PRV recombinants, the *ORF5m* gene (the modified *ORF5* gene having better immune responses) was substituted for the *ORF5* gene and introduced into PRV $TK^-/gE^-/LacZ^+$, resulting in a PRV recombinant named $TK^-/gE^-/GP5m^+$, which expressed the modified GP5m protein. The recombinant virus was confirmed using PCR, Southern blotting and Western blotting. $TK^-/gE^-/GP5m^+$ and $TK^-/gE^-/GP5^+$ expressing the authentic GP5 protein were inoculated into Balb/c mice to evaluate their immune responses. The results indicated that the protecting neutralization antibodies (the 3/6 vaccinated mice obtained 1:16) and cell immune responses induced by $TK^-/gE^-/GP5m^+$ against PRRSV were higher than that induced by $TK^-/gE^-/GP5^+$. Thus, the development of the new PRV recombinant expressing the modified GP5m protein as a candidate vaccine established the basis for the study of bivalent genetic engineering vaccines against PRRSV and PRV.

Keywords porcine reproductive and respiratory syndrome virus, GP5m, the recombinant pseudorabies virus

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1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) caused by the porcine reproductive and respiratory syndrome virus (PRRSV) is a relatively new viral contagious disease affecting severely the swine industry (Meulenberg, 2000). PRRS is characterized by severe reproductive failure in sows, and respiratory distress in piglets and growing pigs. Before the identification of the etiological agent, it was named "mysterious disease". Since the first reported case of PRRS in the United States in 1987 and in Europe and Canada in the subsequent 2–3 years, PRRSV has spread to North American and European continents within several years and has now spread to most Asian countries. Although PRRS was first reported in China in 1995 (Madsen et al., 1998), it is now widespread in China, and has shown an epidemic trend in recent years. The prevention of PRRS is mostly immunoprophylaxis, as in many other viral diseases. Currently, the vaccines against PRRS are mainly attenuated vaccines and inactivated vaccines. Modified live and killed vaccines, the two types of commercial vaccines currently on the market, have inherent drawbacks. Although live vaccines can provide a certain degree of protection against PRRSV, the intrinsic risk of reversion to virulence in farm conditions remains a concern. The outbreak of PRRS in Danish herds, causally associated with the introduction of a modified live vaccine, illustrates this point (Qiu and Tong, 2000). Even though multiple inoculations are not necessary with killed vaccines, they do not always provide herd immunity. Therefore, a new generation of vaccines with higher safety and protective efficacy is required to control this disease.

It has been demonstrated that the GP5 protein encoded by the ORF5 gene is one of the most immunogenic proteins of PRRSV associated with the production of neutralizing antibodies. GP5 plays an important role in the prevention of PRRSV in animals (Weilan et al., 1999; Dea et al., 2000) and has become a good target protein used in the development of a new generation of PRRS vaccines (Gagnon et al., 2003).

To develop a safe and effective vaccine, Fang (2003) even constructed two Pseudorabies virus (PRV) recombinants ($TK^-/gG^-/GP5^+$ and $TK^-/gE^-/GP5^+$) expressing native PRRSV GP5 from an attenuated PRV vaccine strain. Fang and co-workers investigated their immunogenicity in mouse and piglet models, and no detectable neutralizing antibody to PRRSV could be elicited (Fang, 2003; Fang et al., 2004). Recently, Jiang et al. (2005) constructed a modified *ORF5m* gene from the native PRRSV *ORF5* gene, which was generated by introducing a Pan DR helper T cell epitope (PADRE) between the potential neutralization and decoy epitopes. GP5m could induce stronger immune responses and its enhanced immunogenicity was demonstrated in the context of DNA vaccination and displayed a potential application value (Jiang et al., 2005). On account of the above results, we used *ORF5m* as the target gene, and used homologous recombination to construct the PRV recombinant $TK^-/gE^-/GP5m^+$ (RV5m) expressing GP5m. The immunogenicity of RV5m was further compared with $TK^-/gE^-/GP5m^+$ (RV5) in the mouse model and the potential application value of the new candidate vaccine was preliminarily assessed. This study is of significance in developing a recombinant PRV vector vaccine that can effectively prevent and control PRRSV and PRV.

2 Materials and methods

2.1 Viruses, plasmids and cell lines

The PRV mutation $TK^-/gE^-/LacZ^+$ is a PRV Ea derivative in which TK and gE are inactivated. A beta-galactosidase expression cassette was inserted in the gE locus and was used as a parental virus for the construction of recombinant viruses in this study (Liu et al., 2005). In this study, $TK^-/ gE^-/GP5^+$ (RV5) expressing GP5 of the PRRSV YA1 strain was used as a control vaccine to evaluate the immune potency of the recombinant virus RV5m (Fang, 2003). A universal transfer plasmid pIECMV containing the expression cassette driven by the hCMV promoter was used as a backbone to construct the recombinant transfer plasmid. The plasmid *pMD-ORF5m* containing the full-length *ORF5m* gene was constructed and maintained by the authors (Jiang et al., 2005). PRV virulent Ea strain, IBRS-2 cells and *E. coli* DH5α were grown and maintained in our laboratory.

2.2 Primer design and PCR amplification

The *ORF5m* gene, about 650 bp, was PCR amplified using pMD-ORF5m as the template and ORF5A containing a *Bam*HI site (5′-GGA***GGATCC***AGTATGTTGGGGAAATGC-3′) and ORF5B containing an *Xba*I site (5′-TTT***TCTAGA***GACGA-CCCCATTGTTCCG-3′) as forward and reverse primers, respectively. The partial fragment, about 430 bp of the LacZ expression cassette, was PCR amplified using primers LacZP1 (5′-GAACTGCCTGAACTACC-3′) and LacZP2 (5′-ACTGCAACAACGCTGC-3′) to identify the dissemination of the parental virus in RPV recombinants (Liu et al., 2002).

2.3 Construction of the recombinant transfer plasmid

pIECMV carries a *Kpn*I-*Bam*HI fragment of the unique short region of PRV strain Ea, and partial coding regions of gI and gE are replaced by an expression cassette containing the immediate early promoter of human cytomegalovirus (CMV), multi-clone sites (MCS), and bovine growth hormone polyadenylation signal (BGH polyA). To generate the transfer plasmid pIECMV-ORF5m, the modified ORF5m gene was excised from pMD-ORF5m as a *Bam*HI-*Xba*I fragment about 650 bp and inserted into the unique *Bam*HI and *Xba*I sites of pIECMV, resulting in pIECMV-ORF5m.

2.4 Co-transfection and plaque screening

The genomic DNA of the PRV Ea mutant $TK^-/gE^-/LacZ^+$ was extracted and purified as previously described (Liu et al., 2002). Recombinant viruses were generated by co-transfection and homologous recombination as previously described with slight modifications (Qiu et al., 2005). Briefly, before co-transfection, purified genomic DNA was digested with *Eco*RI, extracted with phenol and chloroform, precipitated in cold ethanol, and dissolved in TE (10 mM Tris [pH 8.0], 1 mM EDTA). The linear transfer plasmids and the *Eco*RI-digested genomic DNA were co-transfected into IBRS-2 cells. Co-transfection was performed with Lipofectamine™ 2000 reagent (Invitrogen) as specified by the manufacturer. At 3 days post co-transfection, the virus-containing supernatant was harvested. Transfection progenies were plated onto IBRS-2 cells for performing the plaque purification assay. Possible positive recombinants were screened by their white-plaque phenotype under an agarose overlay containing 300 μg of Bluo-Gal (Invitrogen) per ml. White plaques were picked by aspiration and were purified three times by PCR amplifications of the inserted interest genes (ORF5, ORF5m, ORF6) or the exchanged LacZ gene.

2.5 Identification of the recombinant virus

2.5.1 PCR identification

The positive recombinant virus obtained by the three-generation plaque screening method was further purified once again. Viral white-plaques selected at random were inoculated on IBRS-2 cells on a 24-well plate. Cytopathic cells were lysis and PCR amplified for the target ORF5m and LacZ genes to identify the PRV recombinant and the dissemination of the parental virus.

2.5.2 Southern blotting

The genomic DNA of the PRV recombinant $TK^-/gE^-/GP5m^+$ was extracted and purified as previously described (Qiu et al., 2005). The genomic DNA was restricted with eligible restriction enzymes and electrophoresed for hybridization by Southern blotting with Dig-labeled *ORF5* gene fragments. The procedure was performed according to the instruction manual of the Dig High Prime DNA Labeling and Detection Starter Kit I (Roche).

2.5.3 Western blotting

IBRS-2 cells were infected with recombinant PRV and cells exhibiting 80% cell cytopathic effects (CPEs) were collected. The collected cells were lysed with a suitable volume of cell lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and the lysate was used as antigens in Western blotting. The solubilized proteins were pre-treated for 5 min at 95°C and separated using SDS-polyacrylamide gel electrophoresis (PAGE). Proteins from the gel were transferred to a nitrocellulose membrane and probed with anti-GP5 rabbit serum followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology). Blots were developed using DAB.

2.6 Animal experiments

2.6.1 Immunization and challenge

Five-to six-week-old female BALB/c mice (purchased from the Animal Center, Institute of Medicine, Hubei Province, China) were randomly divided into four groups (six mice each group). Three groups were inoculated intramuscularly with 0.1 mL medium containing 10^5 50% tissue culture infection dose ($TCID_{50}$) of RV5m, RV5 and the control parental PRV ($TK^-/gE^-/LacZ^+$), respectively. One group inoculated with medium without the virus served as negative control. The mice were booster immunized with identical doses 4 weeks later. Serum samples were collected from the retro-orbital plexus at various time points after immunization for performing serological tests. At 10 weeks after primary immunization, mice were sacrificed and splenocytes were harvested for performing the lymphocyte proliferation assay, or mice were challenged by the intra-footpad route with 5×10^5 PFU of PRV strain Ea and monitored daily. Survivals were investigated for 10 days after virus challenge to assess the potency of the PRV recombinant in protecting the animals against PRV.

2.6.2 Specific antibodies against PRV

Serum neutralization test was performed for PRV as previously described (Qiu et al., 2005). Neutralizing activity of sera of vaccinated mice at 3, 6, 8 and 10 weeks post-primary inoculation (PPI) was expressed as the highest serum dilution that completely prevented the replication of virus in cells. PRV-specific IgG antibody responses were detected by indirect ELISA based on the purified PRV virion (Qiu et al., 2005).

2.6.3 Specific immune responses to PRRSV

Serum neutralization tests were performed for PRRSV as previously described (Jiang et al., 2005). Serum samples were heat inactivated at 56°C for 30 min prior to performing the serum neutralization assay. Two-fold serially diluted sera (50 µL) were mixed with an equal volume of 200 $TCID_{50}$ of the PRRSV strain YA1 containing 20% swine complement in 96-well culture plates and incubated for 1 h at 37°C in 5% CO_2. After incubation, 100 µL MARC-145 cell suspension containing 2×10^4 cells was added to each well. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and examined daily for 6 days for the appearance of a PRRSV-specific CPE. The neutralization titers were expressed as the reciprocal of the highest serum dilution in which no CPE was observed. Each sample was run in triplicate.

Lymphocyte proliferation assay was performed using splenocytes from immunized mice, 10 weeks after primary vaccination. Mouse splenocytes from the sterile spleen homogenates of vaccinated mice were isolated (Fang et al., 2004). The splenocytes were plated in 96-well flat-bottom plates at 100 µL/well (2×10^6 cells/mL). Subsequently, 100 µL of medium was added to each well with or without ultraviolet inactivated PRRSV (an extract of PRRSV-infected Marc-145 cells concentrated by ultracentrifugation at 80,000 g for 2 h) and mixed. Each splenocyte or PBMC sample was plated in triplicate. The stimulation index (SI) was used as the parameter to evaluate lymphocyte proliferation responses. SI was calculated as the ratio of the average OD value of wells containing antigen-stimulated cells to that of the wells containing only cells with medium.

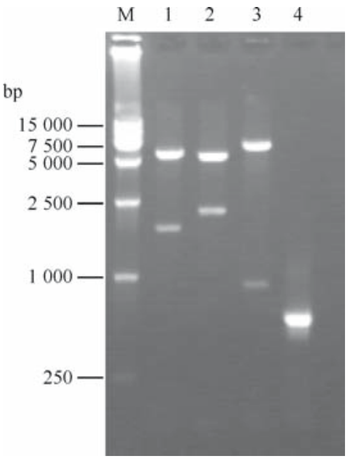
3 Results

3.1 Construction of transfer plasmid

The modified *ORF5m* gene was excised from pMD-ORF5m as a *Bam*HI-*Xba*I fragment (about 650 bp) and inserted into the unique *Bam*HI and *Xba*I sites of pIECMV, resulting in pIECMV-ORF5m. The plasmid pIECMV-ORF5m was identified by restriction and PCR amplification (Fig. 1).

3.2 Screening and purification of recombinant viruses

The structure of the recombinant virus is shown in Fig. 2. The three-generation plaque assay was used to screen for 100% positive recombinants and the positive recombinants were purified one more time by PCR amplification. Cytopathic cells were treated and the target *ORF5m* and the *LacZ* genes were PCR amplified to identify PRV recombinants and to analyze the dissemination of the parental virus, respectively. All plaques were positive to *ORF5m* (about 650 bp) (Fig. 3) and negative to the partial fragment of *LacZ* after PCR



1: pIECMV-ORF5m/*Kpn*I; 2: pIECMV-ORF5m/*Bgl*II; 3: pIECMV- ORF5m/*Stu*I+*Bam*HI; 4: PCR product of *ORF5m* from the plasmid pIECMV-ORF5m

Fig. 1 Identification of the transfer plasmid pIECMV-ORF5m

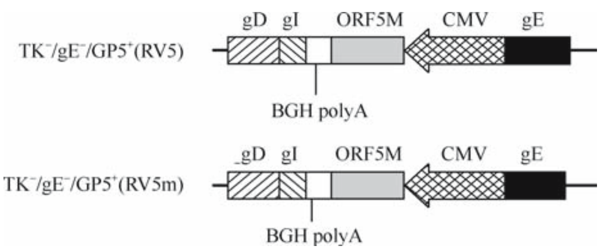
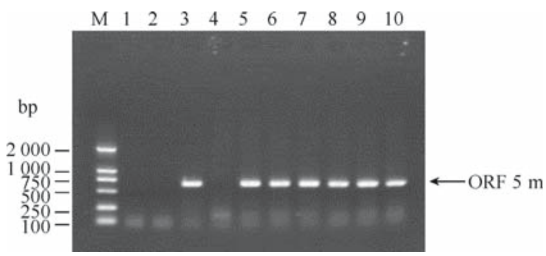


Fig. 2 The pattern structure of the recombinant virus



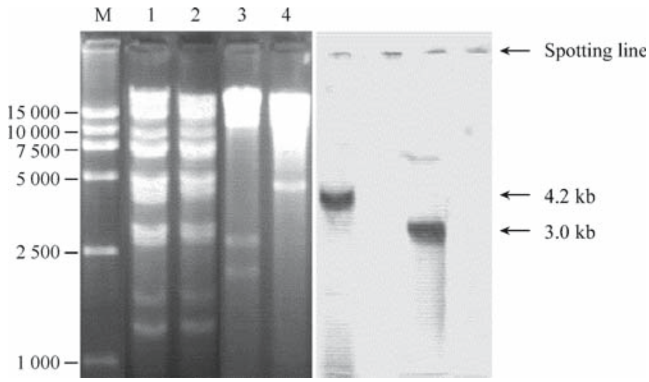
M: DNA marker (DL 2000); 1: Negative control (TK$^-$/gE$^-$/LacZ$^+$); 2: IBRS-2 cell control; 3: Positive plasmid pIECMV-ORF5m control; 4: H2O control; 5–10: the recombinant virus RV5m

Fig. 3 Identification of the recombinant virus TK$^-$/gE$^-$/GP5m$^+$ by PCR

amplification (about 430 bp) (data not shown). And cytopathic cells infected with the parental strain TK$^-$/gE$^-$/LacZ$^+$ were positive to *LacZ* after PCR amplification. The results demonstrated that the recombinant virus contained the specific exogenous gene and did not have the dissemination as that of the parental virus.

3.3 Southern blotting

The correct substitution of partial *gI* gene, *gE* gene and complete *LacZ* gene of TK$^-$/gE$^-$/LacZ$^+$ by the CMV expression cassette expressing GP5m was further verified by Southern blot hybridization using probes specific for ORF5. The genomic DNAs of the PRV recombinant RV5m and the parental virus TK$^-$/gE$^-$/LacZ$^+$ were digested with *Bgl*II or *Sal*I. The genomic DNA of RV5m separately digested with *Bgl*II and *Sal*I showed an expectantly specific band of hybridization about 4.2 kb and 3.0 kb, respectively, and that of digested TK$^-$/gE$^-$/LacZ$^+$ showed no bands of hybridization (Fig. 4). The results indicated that the exogenous gene of the recombinant virus was specifically integrated into the gE and gI sites of PRV.

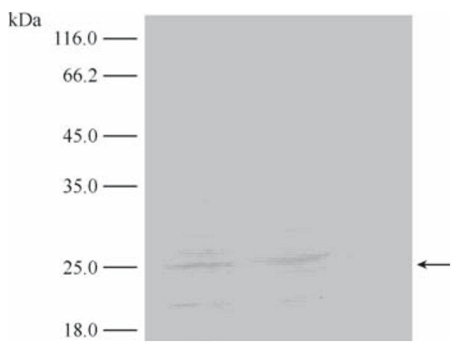


M: DNA marker (DL15000); 1: RV5m genome/BglII; 2: TK$^-$/gE$^-$/LacZ$^+$ genome/BglII; 3: RV5m genome/SalI; 4: TK$^-$/gE$^-$/LacZ$^+$ genome/SalI

Fig. 4 Identification of the recombinant virus by Southern blotting

3.4 Western blotting

To investigate the expression of the exogenous gene in the PRV recombinant, RV5m and TK$^-$/gE$^-$/LacZ$^+$ were inoculated into IBRS-2 cells. Cytopathic cells were treated and analyzed using 12% SDS-PAGE and Western blotting. As shown in Fig. 5, an expectantly specific band, corresponding approximately to 26–27 kDa, was recognized using antiserum and lysates of RV5m-infected cells as antigens. These results indicated that the GP5m protein from the PRV recombinant was expressed, and which biological and immunological properties were similar to that of native GP5m.



1: RV5; 2: RV5m; 3: TK$^-$/gE$^-$/LacZ$^+$

Fig. 5 Western blot analysis of the cell lysate infected with the recombinant virus

3.5 Pseudorabies virus (PRV)-specific serum antibody responses and protection in mice

One of the properties for the successful development of a bivalent vaccine based on a viral vector is that the recombinant vaccine should retain the immunogenicity of the parental virus. All mice inoculated with recombinants (RV5 and RV5m) expressing PRRSV-derived antigens (GP5 and GP5m) or $TK^-/gE^-/LacZ^+$ developed comparable PRV-specific ELISA and neutralizing antibodies at 3, 6, 8 and 10 weeks PPI. The specific, detectable anti-PRRSV neutralizing antibodies were produced in the RV5 and RV5m groups at 3 weeks PPI and reached a peak at 6 weeks PPI. No significant difference was observed among the vaccinated mice except in mice from the negative control group ($P > 0.05$) (Table 1).

Table 1 The PRV-specific antibodies of mice inoculated with the recombinant virus

		3W	6W	8W	10W
$TK^-/gE^-/LacZ^+$	SN[a]	1/6[c]	31.3±18.4	35.6±17.5	34.6±19.7
	ELISA[b]	160	20480	20480	20480
RV5	SN	1/6	28.8±20.9	35.2±17.5	34.8±17.8
	ELISA	160	20480	20480	20480
RV5m	SN	2/6	38.4±24.3	33.6±17.6	35.2±16.5
	ELISA	160	20480	20480	20480
Negative control	SN	–	–	–	–
	ELISA	–	–	–	–

SN is seroneutralization antibodies
PRV ELISA antibodies titers based on PRV virions
The ratio of the vaccinated mice of which the PRV-seroneutralization antibodies were the lowest at 1:2 in each group containing six vaccinated mice.

At 10 weeks PPI, all vaccinated mice were challenged with 10^6 $TCID_{50}$ of PRV strain Ea through the intra-footpad route. Mice inoculated with PRV-derived viruses showed a 100% survival rate. All mice in the negative control group died within 5–9 days post challenge (Fig. 6). These data indicated that insertion of the PRRSV genes in the gE/gI locus did not influence the immunity and protective efficacy of recombinant viruses against PRV.

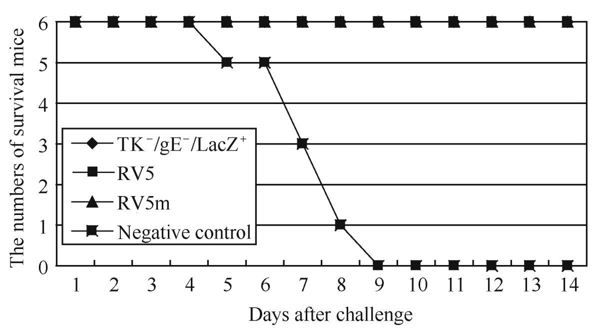


Fig. 6 Protection of mice inoculated with the recombinant virus against PRV challenge

3.6 Porcine reproductive and respiratory syndrome virus (PRRSV)-specific humoral immune responses in mice

Porcine reproductive and respiratory syndrome virus (PRRSV)-specific neutralizing antibodies were detected at 3, 6, 8 and 10 weeks PPI. As shown in Table 2, only one out of six mice inoculated with RV5 expressing native GP5 developed scarcely detectable neutralizing antibodies (1:8) at 10 weeks PPI. Some mice immunized with RV5m developed detectable PRRSV-specific neutralizing antibodies at 6 weeks, and all mice immunized with RV5m developed a peak in the neutralizing antibodies at 10 weeks PPI. Three out of six mice inoculated with RV5m developed 1:16 neutralizing antibodies significantly prior to RV5 inoculation ($P < 0.01$) (Table 2). The results indicated that the PRV recombinant expressing the modified GP5m had higher potential to induce neutralizing antibodies. No detectable PRRSV-specific neutralizing antibodies were produced in the parental virus $TK^-/gE^-/LacZ^+$ and negative control groups during the experiment.

Table 2 The PRRSV-specific neutralizing antibodies of mice inoculated with the recombinant virus

		<1:8	1:8	1:16	1:32
$TK^-/gE^-/LacZ^+$	6W[a]	6[b]	0	0	0
	8W	6	0	0	0
	10W	6	0	0	0
RV5	6W	6	0	0	0
	8W	6	0	0	0
	10W	5	1	0	0
RV5m	6W	5	1	0	0
	8W	1	3	2	0
	10W	0	3	3	0
Negative Control	6W	6	0	0	0
	8W	6	0	0	0
	10W	6	0	0	0

a: Time after primary vaccination, b: Number of vaccinated mice

3.7 PRRSV-specific cellular immune responses in mice

We also detected the lymphocyte proliferative responses of all mice at 10 weeks PPI to analyze the cellular immune responses induced by the PRV recombinant expressing GP5m. As shown in Fig. 7, SI in the RV5m group was significantly

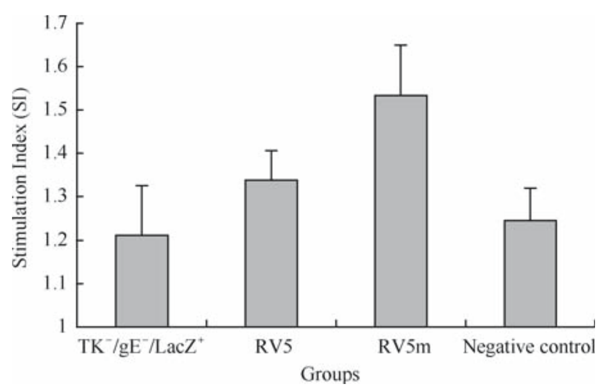


Fig. 7 Lymphocyte proliferative responses of mice vaccinated with recombinant virus vaccines against PRRSV

higher than that in the RV5 group when using ultraviolet-inactivated PRRSV as the stimulatory antigen. The results indicated that the PRV recombinant expressing the modified GP5m had higher potential to induce cellular immune responses.

4 Discussion

Live vaccines based on recombinant viruses represent a particularly promising avenue of vaccine research, both for improving existing vaccines and for developing new ones. PRV, an alpha herpesvirus, is the causative agent of pseudorabies (also known as Aujeszky's disease) in swine. Based on the live attenuated vaccine strain, several PRV recombinants expressing immunogens of heterologous pathogens have had great success in the construction of bivalent or multivalent genetically engineering vaccines (Hooft et al., 1996). At the time of developing an eradication plan for PRV, the vaccinated animals were also vaccinated against other diseases to achieve immunity against two or more diseases. The present study constructed the PRV recombinant RV5m expressing the modified GP5m protein derived from a strong CMV promoter based on the PRV genetically depleted vaccine $TK^-/gE^-/LacZ^+$. The humoral and cellular immune responses of vaccinated mice suggested that RV5m can induce the animals to produce specific neutralizing antibodies and lymphocyte proliferative responses, and would become a new generation of candidate vaccine with potential for further development.

Although the immunological mechanism of PRRSV infection is still unclear, neutralizing antibodies are presumed to play an important role in the first line of defense against PRRSV infection based on current research, and, particularly, against re-infection and the prevention or reduction of viral spread from animal to animal. Osorio et al. (2003) reported that sows, which had acquired a neutralizing antibody titer of 1:16 by passive immunity, could clear the PRRSV infection when challenged at 90 days gestation and did not experience reproductive failure. Similarly, high-titer neutralizing antibodies, which were passively transferred to pigs, were reported to protect them from disease and effectively prevent PRRSV viremia, reported by Yoon et al. (1996). Evenly, the efficacy of protection induced by DNA vaccines against PRRSV had a significant correlation with that produced by neutralizing antibodies (Barfoed et al., 2004). Therefore, neutralizing antibodies had become an important parameter to evaluate the new vaccines of PRRSV. Before the present study, we also constructed a recombinant PRV (RV5) expressing native PRRSV GP5 based on another attenuated PRV vaccine strain ($TK^-/gE^-/LacZ^+$) with the deletion of *TK* and *gE* genes. We investigated its immunogenicity in a mouse model and no detectable neutralizing antibody to PRRSV could be elicited (Fang, 2003; Fang et al., 2004; Qiu et al., 2005). On comparison with the immune responses induced by RV5, one mouse immunized with RV5m developed detectable PRRSV-specific neutralizing antibodies at 6 weeks, and all mice immunized with RV5m developed a peak in the neutralizing antibodies at 10 weeks PPI, in which three out of six mice developed 1:16 neutralizing antibodies. Meanwhile, no significant differences were observed in specific antibodies and protection against PRV between RV5m constructed in this study and the parental virus $TK^-/gE^-/LacZ^+$, which indicates that inserting the heterogeneous gene *ORF5m* in the gE/gI locus does not influence the propagation of PRV and antibody production against it.

Beside the specific neutralizing antibodies against PRRSV, the cellular immune responses produced by RV5m were also significantly superior to that produced by RV5. *ORF5m* is a modified *ORF5* wherein a Pan DR helper T-cell epitope (PADRE) is introduced into the potential neutralization and decoy epitopes, constructed in our previous study (Jiang et al., 2005). The initial objective of this design was to display the neutralization epitope and to minimize the decoy effect of the immunodominant decoy epitope. The enhanced immunogenicity of GP5m was demonstrated in the context of DNA vaccination (Jiang et al., 2005). This is the reason we constructed recombinant PRV expressing GP5m. PADRE, a general Th cell epitope, was constructed by Alexander, which imported the molecular anchor sequence from HLA-DR into a poly-alanine skeleton (Alexander et al., 1998). The capability of PADRE inducing Th cell responses is 1,000 times higher than that of the natural Th epitope. PADRE can strongly influence B cells, CTL epitopes and carbohydrate antigens to induce highly effective Th responses. The predominance of the cellular immune responses of RV5m provided experimental evidence for RV5m as a new promising vaccine against PRRSV.

In summary, we demonstrate here that the PRV-based recombinant virus expressing the modified GP5m protein of PRRSV can develop PRV- and PRRSV-specific immune responses, and is a promising candidate to be further explored for its use as a bivalent vaccine against both PRV and PRRSV infection. Certainly, our present data only demonstrate the potential of such bivalent vaccines in mice. It is necessary to further study whether this vaccine can induce higher immune responses and protection in natural hosts, i.e., pigs, and evaluate comprehensively the all-round efficacy of this vaccine in order to promote its practical application.

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