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Full Length Research Paper

Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing ORF2/ORF1 of PCV2 and P1 2A of FMDV

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A recombinant fowlpox virus (rFPV-ORF2ORF1-P12A) containing the open reading frame ORF2)/ORF1 DNAs of the porcine circovirus 2 (PCV2) (strain, Inner Mongolia) and foot-and-mouth disease virus (FMDV) capsid polypeptide of O/NY00 was evaluated for its abilities to induce humoral and cellular responses in piglets. In addition, we examined its abilities to protect cell cultures against a homologous virus challenge. To approach the feasibility of different united ways of immunization, the recombinant fowlpox virus rFPV-ORF2ORF1-P12A and the recombinant DNA plasmid pVAX1-IL18-ORF2ORF1 were used to immunize the pigs in "prime-boost"programme. We observed that priming the pigs with DNA plasmid pVAX1-IL18-ORF2-ORF1, followed by boosting with the recombinant virus rFPV-ORF2ORF1-P12A produced partially cellular immunity and humoral immunity. Control groups were inoculated with wild-type fowlpox virus (wtFPV) and phosphate buffer saline (PBS). All animals vaccinated with rFPV-ORF2ORF1-P12A developed specific anti-PCV2/anti-FMDV enzyme-linked immunosorbent assay (ELISA) and neutralizing antibodies and also showed T lymphocyte proliferation response. The antibody level produced by PCV2 was lower than that of O type FMDV to 1:20 and 1:200 respectively. We examined specific cytotoxic T lymphocyte (CTL) production in pigs serum and T lymphocytes (CD4, CD8, and CD4/CD8 double positive T cells) in the peripheral blood. First inoculating pVAX1-IL18-ORF2ORF1 and then rFPV-ORF2ORF1-P12A, had considerably higher CD4+, CD8+ and CD4+CD8+ T lymphocytes subgroups compared with the control groups. Whether the ratio between effective cells and target cells was 50:1 or 25:1, the specific CTL of experimental groups had much more significant differences with the control (FPV), even still the group of priming nucleic acid vaccine boosting recombinant virus had the bravest cytotoxicity of specific CTL. Moreover, the E/T ratio of 50:1 was more excellent. Following infection respectively with a mixture of a pathogenic strain of PCV2 (strain, Inner Mongolia)/FMDV (O/NY00) and neutralizing antibody, PK15 cells (BHK21) inoculated with recombinant fowlpox virus (rFPV) showed less ($P < 0.05$) yellow-green fluorescence and cytopathogenesis, suggesting the establishment of partial protection against PCV2/FMDV infection. The results show that the immunization programme here, in which pVAX1-IL18-ORF2ORF1 DNA vaccine was inoculated firstly and rFPV-ORF2ORF1-P12A was followed, is viable and indicates the potential use of a fowlpox virus-based recombinant vaccine for the control and prevention of PCV2/FMDV infections.

Key words: PCV2, rFPV, FMDV, immune response, prime-boost.

INTRODUCTION

The porcine circovirus 1 (PCV1) was initially isolated as a persistent contaminant of the porcine kidney cell line PK-15 (Tischer et al., 1982). PCV1 is a ubiquitous virus that does not cause any disease in piglets (Allan et al., 1995; Tischer et al., 1986). PCV2 is associated with clinical diseases in pigs (Kennedy et al., 2000; Meehan et al.,

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2001).

Post-weaning multisystemic wasting syndrome (PMWS), an emerging disease in pigs, is caused by PCV2 (Ellis et al., 1998; Kennedy et al., 2000; Ellis et al., 1999). This PMWS and other porcine circovirus associated diseases (PCVAD) have caused severe economical losses for swine farmers and the pig industry worldwide. The exact mechanisms of how PCV2 contributes to the clinical manifestation of PCVAD are poorly understood and might include host and viral genetic determinants (Opriessnig et al., 2006), the presence of other infectious agents (Dorr et al., 2007), or environmental factors. However, evidence suggests that its manifestation requires coinfection with a pathogen such as porcine parvovirus (PPV) (Ellis et al., 2000) or a similar immune stimulant (Krakowka et al., 2001), stressor, or cofactor.

PMWS mainly affects 5 to 16 week-old pigs (Allan et al., 2000; Harding and Clark, 1997; Segales et al., 2004). The characteristic clinical signs of PMWS include progressive weight loss, dyspnea, enlargement of lymph nodes, diarrhea, pallor, and jaundice (Allan et al., 2000; Segales et al., 2004). PMWS, a new emerging swine disease worldwide since its first identification in Canada in 1991 (Allan et al., 2000; Chae et al., 2004), is now endemic in many pig-producing countries and causes a potential economical impact on the swine industry worldwide.

PCV is an icosahedral, nonenveloped virus, and measures 17 nm in diameter. The genome of PCV is a single-stranded circular DNA of about 1.76 kb. PCV genome contains at least two potentially functional open reading frames (ORFs): ORF1 (930 bp) encodes the Rep protein involved in viral replication and ORF2 (690 bp) encodes the immunogenic capsid protein.

The overall DNA sequence homology within the PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68 to 76%. Two major open reading frames (ORFs) have been recognized for PCV; ORF1, called the *rep* gene, which encodes a 35.7-kDa protein involved in virus replication (Mankertz et al., 1998), and ORF2, called the *cap* gene, which encodes the major 27.8-kDa immunogenic capsid protein (Cheung, 2003; Nawagitgul et al., 2002; 2000). In addition to the replicase ORF1 and the capsid protein ORF2, the virus genome is predicted to contain another five potential ORFs encoding proteins larger than 5 kDa (Meehan et al., 1997). Whether these potential ORFs are expressed or not and whether the expressed proteins are essential for viral replication remain to be elucidated.

The use of fowlpox virus vectors for heterologous antigen delivery has been explored in a variety of fields (Jiang et al., 2005; Tine et al., 2005; Karaca et al., 2005).Despite the fact that their replication is restricted to avian species, attenuated strains of the fowlpox virus have shown to be efficacious and extremely safe vectors for mammals. The inoculation of fowlpox virus-based recombinants into mammalian cells has in fact resulted in the expression of the foreign gene and induction of protective immunity (Karaca et al., 2005). This observation shows that favorable immune responses could be induced in the absence of productive replication, while eliminating the potential for dissemination of the vector within the vaccinator and the spread of the vector to non-vaccinated contacts or to the general environment (Taylor and Paoletti, 1988; Pastoreta and Vanderplasschen, 2003). However, the use of the fowlpox virus as a vaccine vector for recombinant PCV2 vaccines has not been previously investigated.

In this study, the immune responses of pigs inoculated with a recombinant fowlpox virus vaccine by different vaccination schemes were evaluated. We examined the ability of priming the recombinant fowlpox viruses (rFPV-ORF2ORF1-P12A) and/or boosting the recombinant plasmid (pVAX1-IL18-ORF2ORF1) to elicit PCV2-specific immune responses and protective efficacy in pigs. Our results indicate that the recombinant fowlpox virus, rFPV-ORF2ORF1-P12A, may be an effective vaccine affording protection to pigs against PCV2 challenge.

MATERIALS AND METHODS

Viruses cells and plasmids

The wild type strain of the fowlpox virus (wtFPV) is an attenuated vaccine produced by the Animal Pharmaceutical Factory of Nanjing (Nanjing, China). The recombinant fowlpox virus rFPV-ORF2ORF1-P12A containing the ORF2/ORF1 DNAs of PCV2 (strain, Inner Mongolia) and the foot-and-mouth disease virus (FMDV) capsid polypeptide of O/NY00 was previously constructed in our lab.

wtFPV and rFPV were prepared in chicken embryo fibroblast (CEF) cells in MEM supplemented with 10% fetal calf serum (FCS). PCV2 (strain, Inner Mongolia) originally isolated from lymph node sample of a pig with naturally occurring PMWS (Fenaux et al., 2002) was preserved and passed to PCV-negative PK-15 cells (ATCC CCL33). FMDV O/NY00 was preserved and passed in BHK21 cells to obtain a titer of $1 \times 10^{6.4}$ 50% tissue culture infectious dose ($TCID_{50}$)/0.01 ml in our lab. We previously constructed the recombinant plasmid pUTALP12A encoding the capsid polypeptide genes of FMDV O/NY00 and another recombinant plasmid, pMD18-T-ORF2ORF1, encoding PCV2 genes. The recombinant plasmid (pVAX1-IL18-ORF2ORF1) encoding the ORF2/ORF1 genes of PCV2 and pig interleukin-18 mature protein gene was previously constructed.

Selection and identification of rFPV-ORF2ORF1-P12A

The fowlpox virus shuttle vector pUTALP12A, which was composed of the combined promoter TI-P7.5 (ATI promoter of cowpox virus and 20 tandemly repeated mutant P7.5 early promoters of vaccinia virus) and the *P12A* gene controlled by the single promoter (16 tandemly repeated mutant P7.5 early promoters of vaccinia virus) have been reported previously (Zheng et al., 2006). The plasmid pMD18-T-ORF2ORF1 was digested with *Sma*I to produce an ORF2ORF1 fragment that was then inserted into the *Sma*I site under the combined promoter ATI-P7.5 of pUTALP12A, and the shuttle vector pUTALORF2ORF1-P12A was obtained. Subsequently, the rFPV-ORF2ORF1-P12A was selected and identified as described previously (Zheng et al., Ma et al., 2006). Briefly, the

Table 1. Immunization in "prime-boost" programme.

Group	Code	Inoculated times and biologic	
		1st i. m(1 day)	2nd i. m (28 day)
Controls	N	PBS	PBS
Trials	M	wtFPV	wtFPV
	I	rFPV-ORF2ORF1-P12A	rFPV-ORF2ORF1-P12A
	J	rFPV-ORF2ORF1-P12A	pVAX1-IL18-ORF2ORF1
	K	pVAX1-IL18-ORF2ORF1	rFPV-ORF2ORF1-P12A

shuttle plasmid pUTALORF2ORF1-P12A and wtFPV were cotransfected to 80% confluent CEF cells via liposomes (lipofectamine, Invitrogen Corporation). The viruses were collected after a cytopathic effect (CPE) appeared. They were then screened three times under the presence of 40 µg/mL BudR (5-bromo-2′ deoxyuridine, Sigma) and cultured in MEM medium without BudR. The individual virus plaque was selected, amplified, and purified when CPE appeared, and identified by electron microscope. The purified recombinant virus was named rFPV-ORF2ORF1-P12A.

Experimental animals

We brought 40 segregated early weaned crossbred pigs approximately 8 to 12 week-old pigs from a PCV2- and FMDV-free farm in ChangChun, China.

Inoculation of pigs with rFPV

The pigs were divided into five groups (five pigs per control group and ten pigs per trial group). Pigs in the respective control groups were inoculated intramuscularly with 200 µL phosphate-buffered saline (N) and 2×10^8 plaque-forming units (PFU) of wtFPV (M). Pigs in the trial groups (I J and K) were inoculated intramuscularly with the same dose of rFPV-ORF2ORF1-P12A (2×10^8 PFU) or 200 µg pVAX1-IL18-ORF2ORF1, respectively. All groups were boosted with an equivalent dose at 28 days post-inoculation (dpi) (Table1). Blood samples were collected from each pig pre-inoculation and every week post-inoculation.

Evaluation of the humoral immune response

Evaluation of the humoral immune response to PCV2

Indirect enzyme-linked immunosorbent assays (I-ELISAs) were performed (according to the protocol of the PCV2-Diagnosis-Assay kit provided by the Veterinary College, Huazhong Agricultural University) on pig serum to measure the amount of anti-PCV2 antibodies present in each sample. Briefly, 96-well flat-bottom plates coated with the ORF2 protein of PCV2 were incubated in duplicate with 20-fold dilutions of test sera for 30 min at 37°C. Peroxidase-conjugated anti-pig IgG conjugate was then added for 30 min at 37°C, followed by addition of the substrates (each 50 µl). After 10 min in the dark at room temperature, the color reaction was terminated with 50 µl of dense H_2SO_4, and the absorbance at 630 nm was determined using an ELISA plate reader within 15 min.

Serum samples from all pigs were analyzed for neutralizing antibodies by using a neutralization assay (Tischer et al., 1986) with a monolayer of PK-15 cells. Sera were inactivated at 56°C for 30 min and 2-fold dilutions (in duplicate) and 50 µL of each sample or control serum was added to the well at the end of each row of a 24-well tissue culture plate. Samples were then serially diluted twofold across the plates. Further, 50 µl PCV2 suspension of 200 IU/150 µl (1 IU = amount of virus causing specific immuno-fluorescence in 1 cell/well) was added to each well and the plate was vortexed for 1 min. After incubation at 37°C for 90 min, this mixture was inoculated into 1-day PK-15 cell cultures in each well. After incubation for 1 h (37°C; 5% CO_2), cultures were washed and growth medium (MEM + 5% FCS) was added. 4 h later, cultures were treated with D-glucosamine (Sigma) and thereafter incubated in growth medium. 24 h after inoculation, a period in which PCV2 antigen resulting from a secondary infection of cells by progeny virus has not yet been formed, cultures were fixed and tested for the presence of specifically fluorescent cell nuclei by the indirect fluorescent antibody (IFA) test using rabbit anti-PCV2 antiserum. The neutralizing titer was defined as the highest serum dilution which had caused a 50% reduction in the number of specifically fluorescing cells.

Evaluation of the humoral immune response to FMDV

I-ELISAs were performed (Ma et al., 2008) on pig serum to measure the amount of anti-FMDV antibodies present in each sample. Serum samples from all pigs were analyzed for neutralizing antibodies using a neutralization assay with a monolayer of BHK21 cells (Cedillo-Barron et al., 2001; Yu and 221Cui, 1997). The operating method was the same as described (Zheng et al., 222 2006).

Evaluation of the cellular immune response to immunization

T lymphocyte proliferation assay

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium-bromide) (MTT) assay was performed to determine T lymphocyte proliferation. Peripheral blood (taken at 14 and 42 dpi, respectively) from each pig was collected into tubes containing sodium heparin as an anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample by Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI 1640 medium with 10% FCS. The MTT assays (Zheng et al., 2006) were carried out in triplicate cultures (200 µL 2×10^6 cells/mL) in 96-well flat-bottom plates (Costar). T lymphocytes were stimulated with 2 µg/mL of recombinant VP1 protein, or 1:100 live O/NY00, or 1 µg/mL of Concanavalin A. The plate was incubated at 37°C for 45 h, followed by incubation with MTT for 3 h and addition of 150 µL DMSO to every well to dissolve the deposit. Absorbance was determined at 570 nm and results were expressed as stimulation index (SI) (cpm sample/cpm medium alone).

Analysis of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T lymphocytes subtypes

At 21 and 42 dpi, peripheral blood was collected from each pig. The isolation method of PBMCs was the same as described in previously. PBMCs were adjusted to 1×10^6 cells/mL. After centrifugation, the supernatants were discarded and the remaining cell pellets were resuspended with 50 µL of monoclonal antibodies (MAb) against CD4 or CD8 (PE-labeled anti-pig CD4a and FITC-labeled anti-pig CD8a, BD Biosciences Pharmingen) and incubated on ice for 30 min. After washing, the cells were analyzed with the FACScan cytometer (Becton Dickinson) at an excitation wavelength of 488 nm, 580 nm and 630 nm filters.

CTL assay

At 42 dpi, peripheral blood was collected from each pig. The isolation method of PBMCs was the same as described in above. The PBMCs were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 µg/mL Concanavalin A (Sigma), and 10 U/mL of IL-2 and cultured *in vitro* as the effector cells.

A modified PK15 cell line, which stably expressed the ORF2 protein of PCV2, was previously constructed by transfecting the cells with the mammalian expression plasmid pDisplay-ORF2 (data not shown). The stimulator cells were harvested and treated with 25 µg/mL mitomycin C at 37°C in 5% CO_2 for 2 h. Subsequently, the cells were pelleted and washed four to five times with RPMI 1640 medium. The effector cells (4×10^7 cells) were incubated with stimulator cells at an effector-stimulator ratio of 50:1 or 25:1 for three days at 37°C in 5% CO_2.

To measure the specific lysis of these target cells, the lactate dehydrogenase (LDH) release assay was performed according to the manufacturer's protocol. In 96-well round-bottom plates, target cells were incubated with effector cells at various effector-target ratios for 4 h in phenol red-free RPMI 1640 containing 3% FCS. The supernatant (100 µL/well) was then transferred to 96-well plates, and lysis was determined by measuring LDH release using a non-radioactive cytotoxicity assay kit (Promega Corporation, USA). The absorbance values of the supernatants were recorded at 490 nm on an ELISA microplate reader.

The percentage of specific lysis of PK15 target cells for a given effector cell sample was calculated by the following formula: specific lysis = (OD of experimental LDH release - OD of effector cell spontaneous LDH release - OD of target cell spontaneous LDH release)/(OD of maximum target LDH release - OD of target spontaneous LDH release) ×100%. All determinations were performed in triplicate.

Statistical analyses

Serological responses, $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ subtype T lymphocytes, and SI of inoculated pigs were compared to those of control animals using the analysis of variance and *t*-tests. A P value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Humoral immune response to inoculation with rFPV Specific anti-PCV2 antibody titers (AT) of rFPV-ORF2-ORF1-P12A vaccinated pigs were not significantly different ($P > 0.05$) compared with M/N control groups from 7 dpi to 42 dpi. However, the AT increased gradually after the boost until day 70, while there was no notable difference between the 3 prime-boost immunization regime groups. The amount of specific anti-FMDV antibody in the rFPV-vaccinated groups was significantly increased compared with the M and N inoculated groups. However, the antibody responses of the I, J and K vaccinated groups were not significantly different (Figure 1).

The sera from inoculated pigs were tested for their ability to neutralize PCV2/FMDV in PK-15 cells and BHK-21 cells. At 14 and 42 dpi, sera from rFPV-inoculated pigs showed PCV2/FMDV neutralizing activity (Table 2).

The titer of neutralizing antibodies in rFPV-inoculated pigs ranged from 1:26.6 to 1:46.6 and 1:26.91 to 76.11. The sera from wtFPV-inoculated pigs showed no PCV2/FMDV-neutralizing activity. There was a higher antibody titer of PCV2 in the group primed with rFPV-ORF2ORF1-P12A and boosted with pVAX1-IL18-ORF2ORF1, while the antibody titer of FMDV in the group primed with rFPV-ORF2ORF1-P12A and boosted with rFPV-ORF2ORF1-P12A was the highest.

Cell-mediated immune response

T lymphocyte proliferation response of pigs

Pigs in the rFPV-ORF2ORF1-P12A-vaccinated/ inoculated groups showed a specific T lymphocyte proliferation response (stimulated with live virus or purified recombinant VP1 protein) and a relatively weak non-specific proliferation response (stimulated with ConA). In contrast, pigs in the wtFPV/PBS-inoculated group only developed a weak non-specific T lymphocyte proliferation response. Analysis of the specific T lymphocyte proliferation response showed that the J-inoculated group had a higher SI, but no significant difference was observed between the tested groups (Table. 3).

Analysis of T lymphocytes subtypes

Peripheral blood lymphocytes were analyzed by flow cytometry at 21 and 42 dpi, respectively. At 21 dpi, there were no significant differences ($P > 0.05$) between the percentages of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T lymphocytes of the rFPV-vaccinated groups and that of the wtFPV/PBS-inoculated groups. At 42 dpi, the percentage numbers of $CD8^+$ T lymphocyte subgroups of the rFPV-vaccinated groups were higher ($P < 0.05$) compared with the wtFPV/PBS-inoculated groups. The J-vaccinated group was higher than the other tested groups but no significant difference was observed (Figure 2).

CTL activity of immunized pigs

The cytotoxic activities of the inoculated pigs were

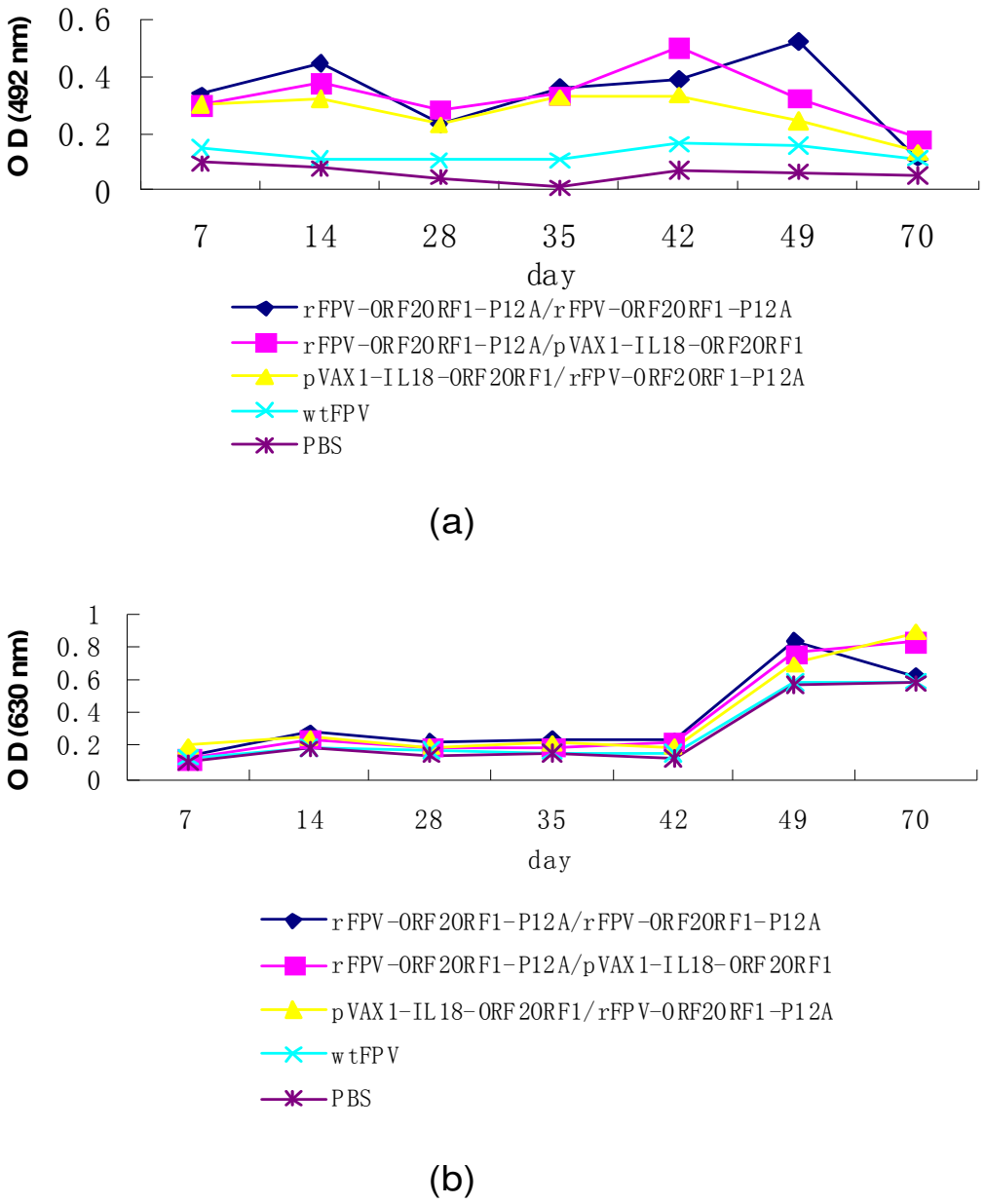

Figure 1. The antibody level of anti-FMDV (a) and anti-PCV2 (b) after immunization. Sera from all pigs were sampled regularly and tested for antibodies against FMDV (1:200 dilution) and PCV2 (1:20 dilution). The results were obtained from the average of the sera in each group.

Table 2. Maximum dilution of neutralizing antibodies in sera of pigs inoculated with rFPV

Immune group	0 dpi		14 dpi		42 dpi	
	FMDV	PCV2	FMDV	PCV2	FMDV	PCV2
I	1.82	1.82	76.11	30.2	76.12	27.0
J	1.82	1.82	38.05	46.6	26.91	40.7
K	1.82	1.82	53.82	26.6	38.05	37.2
M	1.82	1.82	<2	<2	<2	<2
N	1.82	1.82	<2	<2	<2	<2

Neutralizing antibody titers were analyzed at 14 and 42 days post-inoculation (dpi). Serum samples were obtained from five pigs of each group.

Table 3. Specific T lymphocyte proliferation assay.

Group	Stimulate index							
	(Vp1) protein		(O FMDV) live virus		ConA		Control	
	14 days	42 days	14 days	42 days	14 days	42 days	14 days	42 days
I	1.143	1.448	1.888*	1.197	0.941	0.873	0.217	0.39
J	1.337*	1.258	2.078*	1.205	0.988	1.653*	0.287	0.42
K	1.034	1.337	1.889*	1.406	1.017*	1.547*	0.268	0.405
M	0.986	1.225	1.222	1.167	0.914	0.873	0.217	0.39
N	0.927	1.000	1.011	1.000	0.901	1.000	0.193	0.26

T lymphocyte proliferation responses of pigs stimulated by live virus, purified recombinant VP1 protein and ConA, respectively; *represents significant differences.

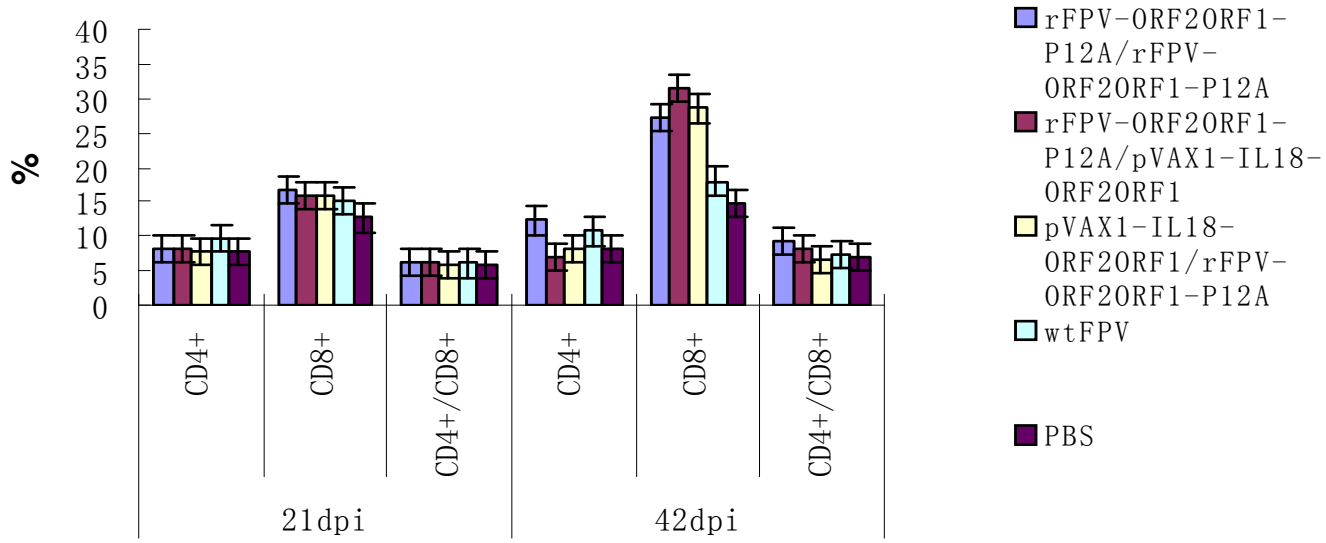

Figure 2. The percentages of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ T lymphocytes of different prime-boost immunization groups. This test was performed at 21 and 42 dpi, respectively. The results were obtained from the average of five pigs sera in each group.

measured by the non-radioactive LDH release assay and the specific lysis rates are shown in Figure 3. All tested vaccines groups elicited specific CTL cytotoxic activities compared with wtFPV- and PBS-inoculated groups ($P < 0.05$). The specific lysis rate of the J or K group was higher than that of the I group.

DISCUSSION

The vaccine potential of recombinant fowlpox viruses is well established. It has been successfully employed in the development of recombinant FMD and of recombinant porcine reproductive and respiratory syndrome (PRRS) vaccines(Zheng et al., 2006; Shen et al., 2007). Recombinant fowlpox virus vaccines offer significant advantages over the traditional virus-inactivated vaccines or recombinant proteins, because the antigen is expressed intracellularly in the immunized animal. Therefore, the processing and presentation of the viral epitope occurs in a way that is similar to a natural infection. Moreover, the fowlpox virus vector is a non-replication vector when used in mammals, so it does not carry the risk of replication associated with Vaccinia virus vaccines (Pastoreta and Vanderplasschen, 2003). Therefore, we attempted to develop a recombinant PCV2 vaccine on the basis on the fowlpox virus vector. In this work, an experimental immunization regimen was developed and tested against PCV2. Piglets coinfected with PCV2 and FMDV developed a more severe clinical disease and PCV2-associated lesions than piglets infected with PCV2 (data not shown). Thus, the gene fragment P12A of FMDV (Berinsteina et al., 2000) in addition to ORF2-ORF1 of PCV2 was chosen and used to construct the recom-binant fowlpox virus. The recombinant fowlpox virus coexpressing ORF2ORF1 (PCV2) and P12A (FMDV) was inoculated in pigs and tested in a protection-challenge experiment, showing that the combination of

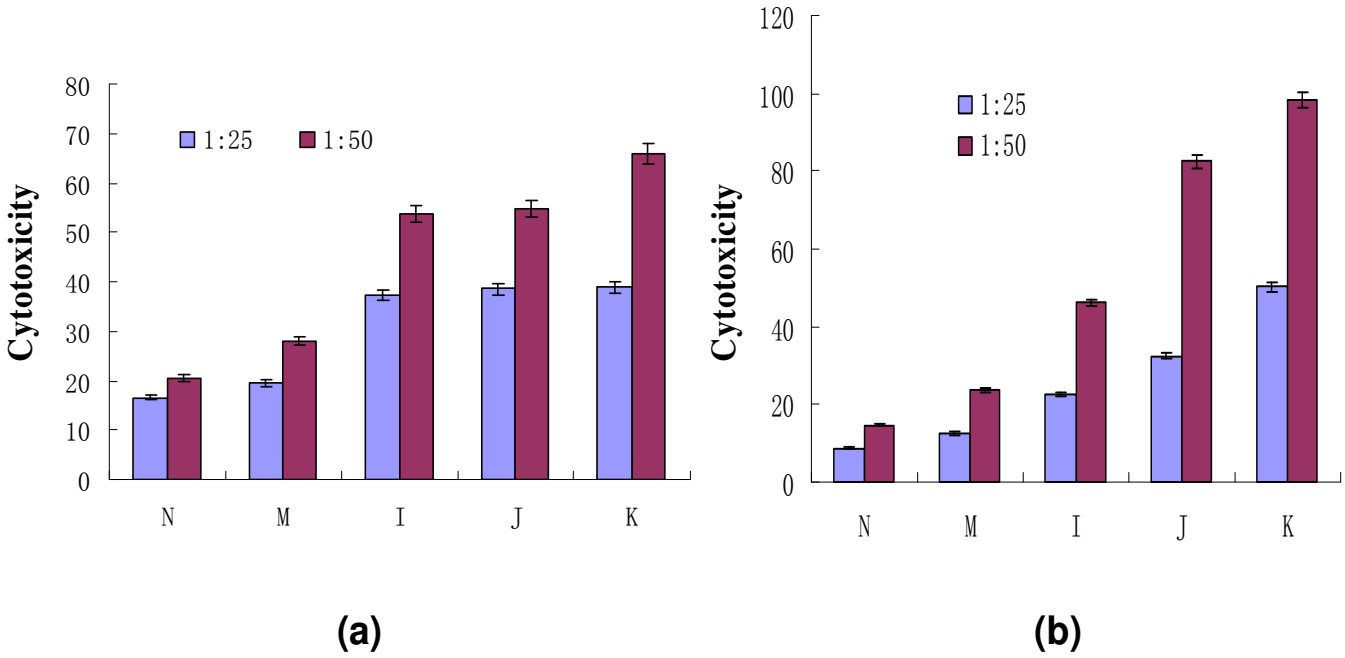

Figure 3. Specific lysis of target cells by restimulated effector cells from the immunized pigs. Effector-to-target cell ratios are indicated in different colors. The percent specific lysis is demonstrated on the vertical axis. This test was performed at 42 dpi. (a) specific CTL activity for PCV; (b) specific CTL activity for FMDV; N=PBS group; M=wtFPV group; I=rFPV-ORF2ORF1-P12A/rFPV-ORF2ORF1-P12A; J = rFPV-ORF2ORF1-P12A/pVAX1-IL18-ORF2ORF1; K = pVAX1-IL18-ORF2ORF1/ rFPV-ORF2ORF1-P12A.

ORF2ORF1 (PCV2) and P12A (FMDV) can induce a partial protective immunity. This finding suggests that this experimental method of antigen presentation may be useful for future protection studies of these and other possibly protective PCV2 (FMDV) antigens, alone or in combination.

A genetic engineering vaccine against PCV2 is not yet available, although an experimental vaccine based on a chimeric virus of PCV1 and PCV2 was very promising (Fenaux et al., 2004; 2003). Engineering vaccine design for manipulating antigen presentation and processing pathways is one of the most important aspect that can be easily handled in the DNA vaccine technology. If an antibody response is the goal, it is clearly desirable to direct antigen expression to the endo plasmic reticulum(ER), in which folding and secretion can occur. An appropriate leader (signal) sequence can achieve this. For induction of CTLs, addition of genes encoding molecules such as ubiquitin, aimed to enhance degradation and peptide production in the proteasome, can be effective. Similarly, targeting expression to different subcellular pathways such as the endosome or lysosome can amplify CD4+ T cell responses.

Vaccination schedules based on combined prime-boost regimens using different vector systems to deliver the desired antigen (that is heterologous prime- boost immunization regimen) appear to be a successful improvement in DNA vaccine platform. Actually, prime-boost regimens have shown promise in eliciting greater immune response in humans compared with DNA vaccination alone (Daniela et al, 2010).

The heterologous prime-boost vaccination regimen exploits the ability of the immune system to generate a large number of secondary antigen-specific T cells. Following a priming immunization, a proportion of the antigen-specific T cell populationtrans forms into antigen-specific memory T cells, which have the ability to expand rapidly up on encounter with the same antigen a second-time round.

Since the priming and boosting vectors are different, and induction of effective immunity are becoming clear, this strategy allows for greater expansion of the disease antigen-specific T cell populations. To date, heterologous prime-boost regimens are among the most potent strategies to induce cellular immune responses. Compared to homologous prime-boost approach with the same DNA vaccine, boosting a primary response with a heterologous vector will result in 4 to 10-fold higher T cell responses (Daniela et al, 2010). Though prime-boost regimens was adopted in the study, the antibody levels in trail groups still had no statistics senses compared with the control ones. So, the recombinant vaccine is to further be improved.

In this study, it is surprising to find that the antibody level of anti-PCV2 was not different from the control (Figure 1b). It is possible that only the four immunodominant regions of ORF2-PCV2 and one of ORF1-PCV2 were determined (Dominique et al, 2000). It

can be thought that ORF1 protein has no immune reactivities. In addition, for the coated ORF2-PCV2 protein used by I-ELISA which was gained by prokaryotic expression, its naturally biological activity may be another factor to affect immune reactivities. Whether the anti-ORF2-PCV2 antiserum generated from a regional PCV2 strain could recognize the same epitopes in strain from other countries is not yet known and such experiments would provide information about epitope variability among PCV2 strains. We found that relatively high levels of neutralizing antibody and protection against PCV2/FMD were induced in all rFPV-vaccinated groups. Pigs inoculated with rFPV developed neutralizing antibodies with titers ranging from 1:26.6 to 1:46.6 (PCV2) and 1:26.9 to 76.11 (FMDV), (Table 1) which indicate that rFPV expressing ORF2ORF1 and P12A induces PCV2- and FMDV-neutralizing antibodies in pigs. Because $TCID_{50}$ was here used to assess the titers of neutralizing antibodies, it is necessary to further examine the result depending on the experimental piglets. Although it is generally accepted that protective immunity to FMDV is principally due to neutralizing antibodies, the cellular immune response provides an essential regulatory role in the induction and expression of the serological response.

In order to evaluate the rFPV-induced PCV2 (FMDV)-specific T cell response, we applied the MTT assay to measure the T lymphocyte proliferation response. Analysis of the specific T lymphocyte proliferation response showed there was a significant difference ($P < 0.05$) between the rFPV-ORF2ORF1-P1 groups and the control groups, and the SI of the prime-boost vaccination with rFPV-ORF2ORF1-P12A and the recombinant plasmid pVAX1-IL18-ORF2ORF1 was higher than in the other groups.

Although a higher SI was achieved with the recombinant VP1 protein, the index was still relatively lower compared with the live viruses. Since wasted pigs always had a lower proportion of the $CD4^+$ cell subsets and a significant downshift of $CD8^+$ or $CD4^+CD8^+$ cells was observed in PCV2-positive pigs (Darwich et al., 2002), the results of T lymphocyte subgroup analysis indicate that at 42 dpi, the percentage numbers of $CD8^+$ T lymphocytes subgroups of the rFPV-vaccinated groups were higher ($P < 0.05$) compared with the wtFPV/PBS-inoculated groups. The percentage numbers of $CD8^+$ T lymphocyte subgroups in the rFPV-ORF2ORF1-P12A/ pVAX1-IL18-ORF2ORF1-vaccinated group was higher than in the other tested groups, but no significant difference was observed (Fig. 2). In addition, at 21 and 42 dpi, the percentage numbers of the $CD8^+$ T lymphocyte subgroups was always higher than that of the $CD4^+$ and $CD4^+CD8^+$ T lymphocyte subgroups. Moreover, priming with rFPV-ORF2ORF1-P12A or boosting with the recombinant plasmid pVAX1-IL18-ORF2ORF1 led to an augmented $CD8^+$ response. In addition, the percentage numbers of $CD4^+CD8^+$ T lymphocyte subgroups was higher at 42 dpi than at 21 dpi. This finding demonstrate that rFPVs have the ability to increase cytotoxic responses and improve memory and/or effector cell responses, although the percentage of $CD4^+CD8^+$ T lymphocytes subgroups were positively related to the pigs' growth .

The CTL assay resulted in statistically noticeable augmentation of specific-CTL activity (PCV2/FMDV) in rFPV-vaccinated groups compared to wtFPV/PBS at 25:1 and 50:1 effector-to-target ratios. This finding also elicited that rFPV-ORF2ORF1-P12A indeed promoted the Th2-mediated immune response consistent with the $CD8^+$ cell subsets being mainly responsible for cytotoxic responses. Additional evidence has been indicated because prime-boost with the recombinant plasmid pVAX1-IL18-ORF2ORF1 and rFPV-ORF2ORF1-P12A at a 50:1 ratio for both PCV2 and FMDV showed a statistically significant increase in the CTL-specific immunogenicity over the other two regimen. Altogether, it is clear that the cellular immune response of rFPV-ORF2ORF1-P12A were better than the antibody titers for PCV2, but rFPV-ORF2ORF1-P12A induces good neutralizing antibodies that is beyond what is expected. Although results of the immune response and viral challenge show that the rFPV-ORF2ORF1-P12A-inoculated group developed a stronger immune response and better protection, it would be advisable to focus future studies on how PCV2 acts on the immune systems of infected pigs. This would allow a more effective use of rFPV-ORF2ORF1-P12A by different inoculation schemes. In summary, in this study, we show that pigs immunized with rFPV-expressing ORF2/ORF1 and P12A were able to elicit a partial humoral and cellular response. Most importantly, a degree of protection was also shown against the challenge with a pathogenic strain of PCV2/FMDV. Moreover, a heterologous prime-boost (rFPV-ORF2ORF1-P12A/pVAX1-IL18-ORF2ORF1 or pVAX1-IL18-ORF2ORF1/rFPV-ORF2ORF1-P12A) immunization strategy was more efficient in inducing a T cell response than the homologous prime-boost (rFPV-ORF2ORF1-P12A/rFPV-ORF2ORF1-P12A). In the PCV2/FMDV $TCID_{50}$ assay, pigs immunized with a heterologous prime-boost regimen such as rFPV-ORF2ORF1-P12A/pVAX1-IL18-ORF2ORF1 showed better protection. These studies demonstrate that this vaccination strategy may be useful and rFPV-ORF2ORF1-P12A could be further developed as a new genetically engineered vaccine.

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