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Interference of porcine circovirus type 2 ORF2 immunogenicity by ORF1 and ORF3 mixed DNA immunizations in mice

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ABSTRACT

Little is known about the influences of other porcine circovirus type 2 (PCV2) proteins on the immunogenicity of Cap protein. Here we constructed plasmids expressing the ORF1 (pORF1) and ORF3 (pORF3) of PCV2, and mixed either of them with the plasmid expressing ORF2 (pORF2) as combined DNA vaccines, to compare their immunogenicity and protective efficacy. Our data revealed that pORF1 reduced the Cap-specific $CD8^+$cell frequency, and both pORF1 and pORF3 attenuated the Cap-specific Th1 and post-challenge-recall VN antibody responses induced by the pORF2 plasmid, despite successful induction of Rep and ORF3 antibodies by pORF1 and pORF3, respectively. Subsequently, protocols with pORF1 or pORF3 showed significantly decreased protective efficacy compared to pORF2 alone. Overall, our data suggested that the ORF1- and ORF3-encoded Rep and ORF3 proteins may interfere with the cellular, humoral and protective immunity of the ORF2-encoded Cap protein *in vivo*.

Introduction

Porcine circovirus (PCV) is a nonenveloped, single-stranded, circular DNA virus with a diameter of 17 nm (Tischer et al., 1982). PCV was first discovered as a noncytopathic contaminant of the porcine kidney cell culture PK-15 (Tischer et al., 1974). The PK-15-derived PCV, designated PCV1, did not produce clinical disease in experimentally inoculated pigs and was considered to be nonpathogenic (Allan et al., 1995; Tischer et al., 1986). In contrast, PCV2 was identified as the primary etiological agent of an emerging disease in 1991, named postweaning multisystemic wasting syndrome (PMWS) (Allan et al., 1998; Allan and Ellis, 2000; Clark, 1997). PMWS is considered an important swine disease and has had a serious economic impact on the global swine industry. This disease affects pigs from 5 to 12 weeks of age, with 5–30% morbidity. Clinical signs of the disease include progressive weight loss, difficult breathing, dyspnea, and jaundice (Clark, 1997). In addition to PMWS, PCV2 is also associated with pneumonia, enteritis, reproductive failure, porcine dermatitis and nephropathy syndrome (PDNS) and a variety of other manifestations (Opriessnig et al., 2007).

The complete genomic sequences of PCV1 and PCV2 have been determined (Hamel et al., 1998; Meehan et al., 1997, 1998; Zhou et al., 2006). The overall DNA sequence homology within PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68 to 76%. PCV2 possesses 3 confirmed ORFs: ORF1 located on the viral plus-strand, ORF2 and ORF3 on the counter-clockwise strand, with lengths of 945, 702 and 315 nts, respectively. PCV2 ORF1 encodes a 35.7 kDa replication protein (Rep) involved in virus replication (Mankertz et al., 1998). PCV2 ORF2 encodes a 27.8 kDa capsid protein (Cap) involved in viral immunogenicity (Mahe et al., 2000; Nawagitgul et al., 2000; Truong et al., 2001). PCV2 ORF3 protein is not essential for PCV2 replication, but involved in PCV2-induced apoptosis (Liu et al., 2005).

As the primary immunorelevant protein, PCV2 Cap protein expressed in insect cells (Nawagitgul et al., 2000) or *Escherichia coli* (Zhou et al., 2005a) could be detected by sera of pigs experimentally infected with PCV2. In addition, multiple immunoreactive regions (Mahe et al., 2000) and epitopes (Lekcharoensuk et al., 2004; Shang et al., 2009) have been identified. Monoclonal antibodies against Cap protein show neutralizing activity against PCV2 (McNeilly et al., 2001; Zhou et al., 2005a), suggesting the protein contains at least 1 neutralizing epitope on the virus. In contrast to Cap, limited information is available on the immunogenicity of Rep and ORF3 proteins. Utilizing PEPSCAN analysis, one immunoreactive area (aa 185–211) was identified in PCV2 Rep protein (Mahe et al., 2000). In another study, 2 immunoreactive T lymphocyte epitopes in Rep protein (aa 81–100 and aa 201–220) and 1 in ORF3 protein (aa 31–50) were demonstrated (Stevenson et al., 2007).

PCV2 Cap protein has been studied intensely as vaccine antigen due to its excellent immunogenicity (Blanchard et al., 2003; Fan et al.,

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2008; Fenaux et al., 2003; Kamstrup et al., 2004; Song et al., 2007; Wang et al., 2006). By using ORF2-based DNA and subunit vaccines in mice, we previously demonstrated that Cap-specific $CD8^+$ T cells and virus-neutralizing (VN) antibody correlating mainly with IgG2a play crucial roles in protective immunity against PCV2 (Shen et al., 2008). In contrast, Rep and ORF3 proteins are generally considered weakly immunogenic and seldom used as vaccine antigens. At this time, their immunorelavent characteristics *in vivo* remain unclear. Since Rep or ORF3 antigens alone may be insufficient to provide adequate immunity against PCV2, combining them with Cap would be an alternative approach to examine their effects on viral immunogenicity. Based on this hypothesis, using a mixed DNA vaccine strategy, we investigated the influences of ORF1 and ORF3 plasmids on the immunogenicity of the ORF2 plasmid. Our results suggest that ORF1 or ORF3 DNA vaccines reduce the immunogenic properties and efficacy of ORF2 DNA vaccines. Our data help elucidate the effect PCV2 Rep and ORF3 proteins play in the onset of immunity in the host.

Results

In vitro expression of mammalian expression vector

Recombinant plasmids expressing PCV2 ORF1 and ORF3 were constructed for use as DNA vaccines. Plasmids were confirmed by PCR, restriction enzyme digestion, and DNA sequencing. *In vitro* expression of protein was analyzed by transient transfection followed by immunoperoxidase monolayer assay (IPMA). When detected with swine PCV2-positive serum, strong signals in pORF1-transfected cells but lacking in pORF3-transfected cells were observed (Fig. 1). However, both of the expressed proteins, localized in nuclei of transfected cells, reacted strongly with the Rep or ORF3 antisera respectively (Fig. 1). As controls, signals were not observed in cells transfected with pCI-neo vector (Fig. 1). The results indicate that the recombinant vectors, pORF1 and pORF3, may express the Rep and ORF3 proteins respectively *in vivo*.

Table 1
PCV2 Cap-specific lymphoproliferative response and FCM analysis of mice splenocytes.

Group	SI	Immunophenotypic cells	
		$CD4^+$ (%)	$CD8^+$ (%)
pORF2 + pCI	1.54 ± 0.32^a	15.80 ± 0.67^a	14.94 ± 2.56^a
pORF2 + pORF1	1.38 ± 0.13^a	14.00 ± 2.87^a	11.58 ± 2.41^b
pORF2 + pORF3	1.36 ± 0.20^a	13.07 ± 1.75^a	$12.08 \pm 1.83^{a,b}$
DNA control	1.00 ± 0.00^b	9.40 ± 3.80^b	11.63 ± 2.17^b

[a, b]Different superscripts within columns represent significant differences between groups ($P<0.05$).

Cap-specific cellular immune responses

The Cap-specific lymphoproliferative responses, $CD4^+$ and $CD8^+$ cell frequencies of vaccinated mice were determined and compared at 8 weeks post the first immunization (p.i.). As shown in Table 1, splenocytes from all the vaccine groups showed proliferative responses when compared to the control mice, with greater statistical difference in pORF2 + pCI group ($P<0.01$) than the pORF2 + pORF1 ($P<0.05$) and pORF2 + pORF3 ($P<0.05$) groups. For flow cytometric analysis, compared with the control group, significantly higher proportions of $CD4^+$ cells were observed in pORF2 + pCI ($P<0.01$), pORF2 + pORF1 ($P<0.01$) and pORF2 + pORF3 ($P<0.05$) groups; however, in the case of $CD8^+$ cells, only the pORF2 + pCI group had a significantly higher frequency than the control ($P<0.05$) (Table 1). Comparing among the vaccine groups, the $CD8^+$ cells in $pORF2^+$ pORF1 group was significantly lower than that in $pORF2^+$ pCI group ($P<0.05$), suggesting a suppressive effect of pORF1 plasmid to the Cap-specific $CD8^+$ cell frequency induced by the pORF2 plasmid.

Total IgG antibody response to PCV2 Cap protein

Total IgG antibody titers against Cap protein were compared among groups. Plasmids pORF2 + pCI elicited the highest antibody response at 4–16 weeks p.i., with the peak IgG titer of 11.2 ± 1.3 log2 at 12 weeks (Fig. 2A). As for the co-administration groups, the combined use of pORF1 and pORF3 induced peak titers of 5.6 ± 3.8

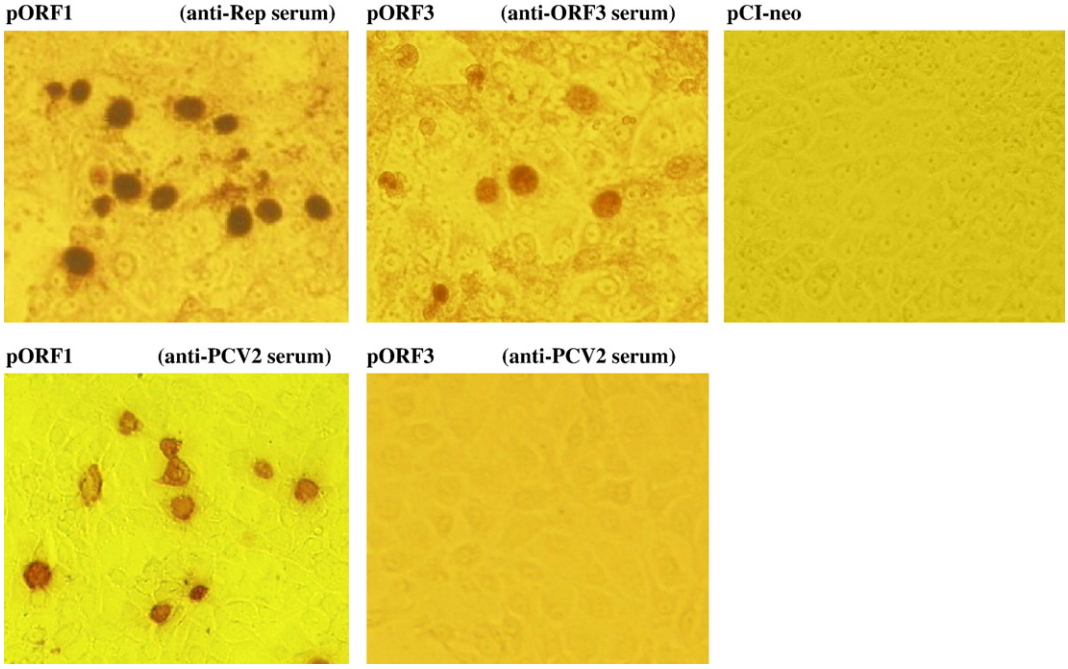

Fig. 1. Expression of PCV2 Rep and ORF3 proteins *in vitro*. PCV-free PK-15 cells were transfected with pORF1, pORF3 or pCI-neo, fixed at 48 h post-transfection, and detected by IPMA. The plasmid and antibody (in parentheses) used for each transfection and detection are indicated on top of each panel; cells transfected with pCI-neo were detected with mouse anti-Rep, rabbit anti-ORF3, and swine anti-PCV2 sera separately.