A RECOMBINANT FUSION PROTEIN AND DNA VACCINES AGAINST FOOT-AND-MOUTH DISEASE VIRUS TYPE ASIA 1 INFECTION IN GUINEA PIGS

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Summary. – On the basis of amino acid (aa) sequence of the tandem repeat 133–158~20–34~133–158 which consisted of aa 133–158 of VP1 and aa 20–34 of VP4 of Foot-and-mouth disease virus (FMDV) type Asia 1 a recombinant prokaryotic expression vector pAS1-P encoding a fusion protein and eukaryotic expression vectors pAS1-E and pAS1-EΔCpG-ODN representing DNA vaccines were constructed. Guinea pigs immunized with these vaccines showed both neutralizing antibody and T cell proliferation responses. FMDV challenge tests for the first time showed that the recombinant fusion protein and pAS1-E and pAS1-EΔCpG-ODN vaccines protected 86%, 60% and 43% of guinea pigs from FMDV type Asia1 challenge, respectively. The results also indicated that the immune response of animals treated with the vector pAS1-E containing an oligodeoxynucleotide (ODN), which consisted of immunostimulatory cytosine-phosphate-guanosine (CpG) motifs, was augmented by CpG ODN.

Key words: Foot-and-mouth disease virus; type Asia 1; DNA vaccine

Introduction

FMDV causes an important disease in cloven-hoofed animals including cattle, sheep, goat and swine (Bachrach, 1968; Kitching, 1999). FMDV is classified as an aphthovirus, a member of the *Aphtovirus* genus, the *Picornaviridae* family. The FMDV genome consists of linear single-stranded positive-sense RNA of an approximate size of 8.5 kb containing a long ORF that codes for a polyprotein which is processed by virus enzymes into mature structural and non-structural proteins (Chinsangaram *et al.*, 1998). The virus exists in the form of seven different serotypes: A, O, C, Asia 1, and South African Territories 1 (SAT1), 2 (SAT2), and 3 (SAT3) and multiple subtypes (Knowles and Samuel, 2003).

Efficient vaccines currently used against foot-and-mouth disease (FMD) are based on inactivated virus (Barteling and Vreeswijk, 2003). However, chemically inactivated virus vaccines have raised safety concerns, as they had been found to be involved in outbreaks of FMD caused by the release of incompletely inactivated virus (King *et al*., 1981). To produce a safer FMDV vaccine, we developed a recombinant fusion protein and corresponding DNA vaccines. Protection against FMD is associated with the induction of neutralizing antibodies. Therefore, attempts have been made to identify peptides capable of eliciting protective humoral responses. Among the structural proteins (VP1 to VP4) of FMDV, VP1 is a capsid protein most exposed on the surface of the virion and carries critical antigenic determinants (Acharya *et al*., 1989; Tulasiram and Suryanarayana, 1998). However, the immunogenicity of the

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Abbreviatios: aa = amino acid; APC = antigen-presenting cell; ConA = concanavalin A; CpG-ODN = oligodeoxynucleotide (ODN) containing CpG motifs; FMD = foot-and-mouth disease; FMDV = Foot-and-mouth disease virus; HCMV = Human cytomegalovirus; IL-12 = interleukin-12; i.m. = intramuscular(ly); IFN-α = interferon-α; SI = stimulation index; TLR9 = toll-like receptor 9; TNF-α = tumor necrosis factor α

VP1 protein isolated from either the virus or the same protein expressed in *Escherichia coli* cells by DNA recombinant techniques is very low compared to the intact virus particle (Kleid *et al.,* 1981). An alternative approach to increase the immunogenicity of FMDV resides in use of biosynthetic peptides containing B and T cell epitopes.

In our previous study we analyzed the sequences of VP1 regions of YNAs1.1 and YNAs1.2 strains of FMDV type Asia 1. Then, according to the nucleotide sequence of VP1 and the reported nucleotide sequence of VP4 of FMDV type Asia 1, a prokaryotic expression vector pAS1 that encoded a fusion protein of β-galactosidase and a tandem repeat in B and T cell epitopes 133–158~20–34~133–158 of FMDV type Asia 1 was constructed. The recombinant fusion protein was found to be immunogenic (Zhang *et al.*, 2002). This result implied that the aa tandem repeat mentioned above could serve as a candidate for fusion epitopes for a vaccine designed against FMD type Asia 1.

In this study, on the basis of the aa tandem repeat, a recombinant prokaryotic expression vector pAS1-P encoding a fusion protein and recombinant eukaryotic expression plasmids pAS1-E and pAS1-EΔCpG-ODN representing DNA vaccines were constructed. The immune responses of guinea pigs immunized with these vaccines were followed. Protection of guinea pigs immunized with these vaccines against a challenge with FMDV type Asia 1 were tested. Finally, the enhancement of immunity by synthetic oligodeoxynucleotide (ODN) containing cytosine-phosphate-guanosine (CpG) motifs (CpG-ODN) was evaluated.

Materials and Methods

Virus. The FMDV type Asia 1 Menglian strain (Zhang *et al.*, 2002), used in challenging guinea pigs, underwent 9 passages in guinea pigs.

Construction of prokaryotic expression vector pAS1-P. In order to increase expression of the epitope protein, the prokaryotic expression vector pAS1-P was derived from pAS1 (Fig. 1, Zhang *et al.*, 2002). The only difference between pAS1-P and pAS1 was that the DNA fragment coding for the tandem repeat 133–158~20–34~133–158 in pAS1-P was synthesized with preference codons in *E. coli,* while that in pAS1 was synthesized with preference codons in the original virus.

Construction of secreted eukaryotic expression vectors pAS1-E and pAS1-EΔCpG-ODN. Using eukaryotic preference codons, the DNA fragments coding for aa 133–158 of VP1 and aa 20–34 of VP4 of FMDV type Asia1 were synthesized and ligated into a tandem repeat with *Eco*RI (5'-end) and *Bam*HI (3'-end) sites (5'-*Eco*RI-133–158~20–34~133–158-*Bam*HI-3'). The Kozak sequence (Kozak, 1987) and the signal peptide of murine IgG were synthesized, fused to the 5'-end of the tandem repeat, and the CpG-ODN fragment was fused downstream the terminal codon of the tandem repeat. Then, this whole fragment was cloned into the eukaryotic expression vector pHook-2 (Invitrogen), thus forming a secreted expression vector pAS1-E (Fig. 2). The latter was a derivative of the vector pAS1-E with the CpG-ODN sequence deleted with *Bam*HI. The structure of all the three recombinant vectors was confirmed by restriction analysis, amplification by PCR and nucleotide sequencing.

The Kozak sequence (underlined) fused to the sequence of signal peptide of murine Ig (in parentheses) was as follows: 5'-*Hind*III-GCCACC(ATGGAGACAGACACACTCCTGCTATGG GTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGAC-*Eco*RI-3'. There is an overlapping ATGG sequence between them. The sequence of CpG-ODN was as follows: 5'-*Bam*HI-TCAACGTT GCTAGACGTTAGCGTGCGCCATCAACG TTTCAACGTTG-*Bam*HI-3'.

Vaccination with the fusion protein and the vectors pAS1-E and pAS1-EΔCpG-ODN. The fusion protein was expressed, identified by Western blot analysis and purified as described previously (Zhang *et al.*, 2002). For vaccination, the fusion protein was emulsified with the adjuvant Montanide ISA 206 (1:1) (Seppic, France). Vectors pAS1-E and pAS1-EΔCpG-ODN were isolated and purified using the Wizard DNA Purification System (Promega) and were dissolved in 0.9% NaCl to a concentration of 1 mg/ml. Asia1 antibody-free guinea pigs were initially injected with 400 μg of the fusion protein, 200 μg of supercoiled pAS1-E and 200 μg of supercoiled pAS1-EΔCpG-ODN, respectively, and boosted 2 weeks later with an equal amount of the fusion protein and DNA by intramuscular (i.m.) route. Three weeks after the last vaccination, the blood was taken and allowed to clot for 30 mins at 37°C. The clots were allowed to contract overnight at 4°C and the sera were harvested, clarified by centrifugation and stored at -20°C.

Titration of neutralizing antibodies. The sera from immunized guinea pigs were inactivated at 56°C for 30 mins and their serial twofold dilutions were prepared. The titration was performed in 96-well plastic microtiter plates in a standard way. The virus dose contained 100 $TCID_{50}$ in 50 μl per well. CPE was read after 72 hrs of incubation at 37°C in 5% CO_2 atmosphere and the titer was expressed as the reciprocal of the serum dilution that resulted in the neutralization of the virus activity by 50%.

Assay of T cell proliferation in immunized animals was carried out as previously described (Zhang *et al.*, 2002).

FMDV challenge test in immunized animals. The ID_{50} of the stock FMDV type Asia 1 was determined by i.m. injection of guinea pigs as described (Zhang *et al*., 2002). Three weeks after the last vaccination dose, the guinea pigs were challenged with 100 ID_{50} of FMDV type Asia 1 in 0.2 ml administered i.m. The animals were monitored for 7 days after the challenge for the appearance of FMD symptoms, such as increase in body temperature above 41°C and appearance of blisters on the footpads.

Results

Antibody response of immunization

Guinea pigs were immunized with purified preparations of the fusion protein and the vector constructs (DNA vaccines) to see whether the fusion protein and tested DNA vaccines were able to elicit a specific antibody response.

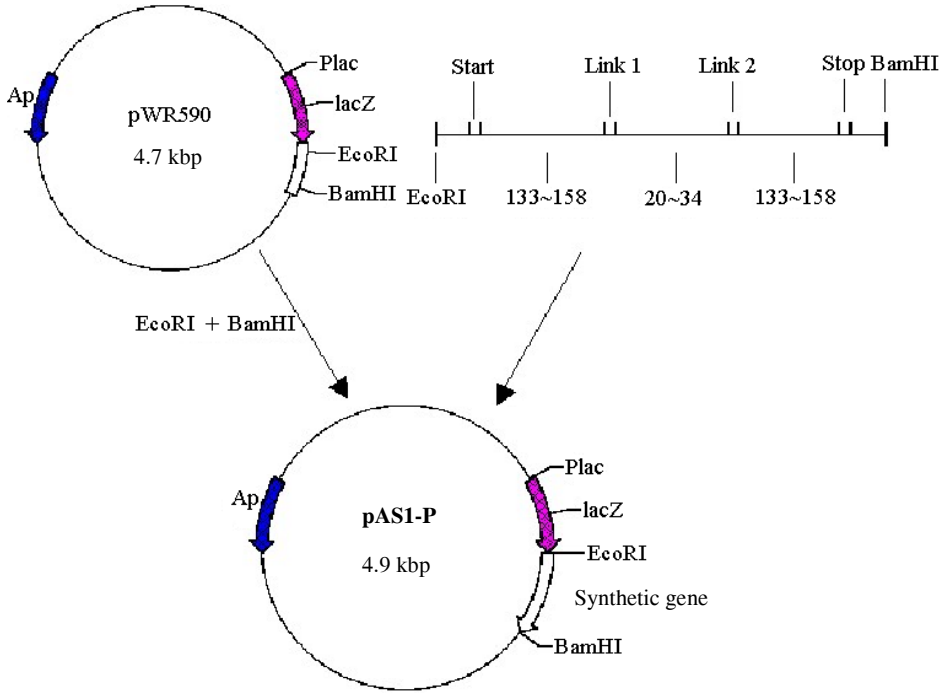

Fig. 1
Construction of secreted eukaryotic expression vector pAS1-E

P_{cmv} = promoter of HCMV; signal = signal peptide sequence of murine Igę chain; Kozak = Kozak sequence; CpG-ODN = oligodeoxynucleotide (ODN) containing CpG motifs.

The results (Table 1) showed that whereas the fusion protein of pAS1-P elicited a high neutralizing antibody response, the vectors pAS1-E and pAS1-EΔCpG-ODN elicited a relatively low neutralizing antibody response. The pAS1-E elicited a higher neutralizing antibody response than pAS1-EΔCpG-ODN.

T cell proliferation response of immunization

To determine the possible cellular response to immunization, T cell proliferation in the immunized animals was followed.

Table 1. Neutralizing antibody titers in guinea pigs immunized with the fusion protein and DNA vaccines

Vaccine tested	No. of animals	Titer of neutralizing antibodies
Fusion protein of pAS1-P	7	420 (256, 128, 246, 512,1024, 256, 512)
pWR590 (control)	5	0 (all 0)
pAS1-E	5	46 (16, 64, 32, 64, 64)
pAS1-EΔCpG-ODN	7	23 (32, 16, 32, 16, 16, 32, 16
pHook-2 (control)	0	0 (all 0)

The results shown in Fig. 2 indicated that T cells proliferated in the guinea pigs immunized with fusion protein of pAS1-P and vectors pAS1-E and pAS1-EΔCpG-ODN. T cell proliferation in the guinea pigs immunized with pAS1-E was slightly stronger than that in the guinea pigs immunized with pAS1-EΔCpG-ODN.

Protection of immunized animals against challenge

Table 2 presents data on the ability of the fusion protein of pAS1-P and DNAs of pAS1-E and pAS1-EΔCpG-ODN to induce immunity in guinea pigs. Eighty-six % (6/7) of the guinea pigs vaccinated twice with 400 μg of the fusion protein of pAS1-P were protected from virus infection; 60% (3/5) and 43% (3/7) of the guinea pigs vaccinated with pAS1-E and pAS1-EΔCpG-ODN were protected from virus infection, respectively. The protection ability of pAS1-E was stronger than that of pAS1-EΔCpG-ODN.

Discussion

In our previous study (Zhang *et al.*, 2002), we demonstrated that the tandem repeat 133–158~20–34~133–158 was

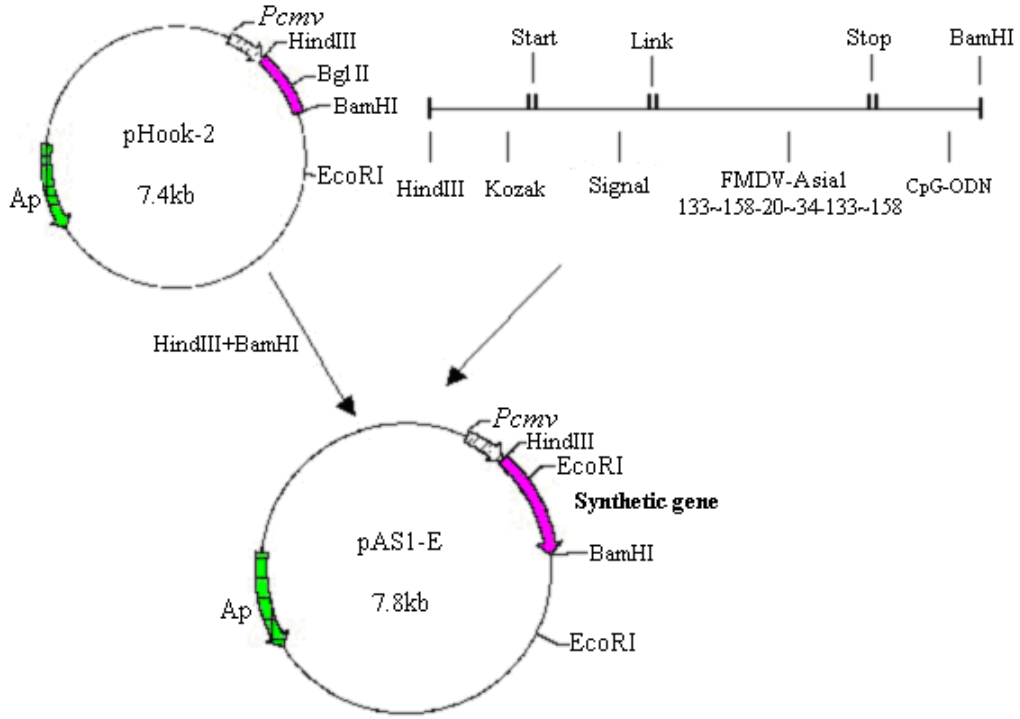

Fig. 2
Construction of prokaryotic expresion vector pAS1-P
For the abbreviations see Fig. 1.

immunogenic. In this study, in order to increase the expression of the epitope protein, the expression vector pAS1-P was derived from the vector pAS1 in which the DNA fragments coding for the tandem repeat 133–158~20–34~133–158 was synthesized with preference codons from *E. coli*. The results showed that the expression of pAS1-P increased by 30%, while that of pAS1 only by 15%. Because of good traits of a DNA vaccine, such as the safety and convenience in manufacturing, storage, and administration, and a potential to elicit both cell-mediated and humoral immune responses (Donnelly *et al.*, 1997; Tighe *et al.*, 1998), the secreted eukaryotic expression vectors pAS1-E and pAS1-EΔCpG-ODN (the DNA vaccines) were also constructed. The results showed that the fusion protein of pAS1-P and the DNA vaccines elicited neutralizing antibodies in guinea pigs, and the T cell proliferation in animals immunized with these vaccines increased following stimulation with the FMDV type Asia 1 antigen. Then, FMDV challenge experiments showed that the recombinant fusion protein and the vaccines protected 86%, 60% and 43% guinea pigs from FMDV type Asia 1 challenge, respectively. These results further confirmed that the tandem repeat 133–158~20–34~133–158 was immunogenic. Because the recombinant fusion protein of pAS1-P exerted a solid protection and the peptide of aa 20–34 of VP4 of FMDV type Asia 1 was an important T cell epitope in cattle (van Lierop *et al.*, 1995), the results imply that the recombinant fusion protein vaccine of pAS1-P may be effective against the circulating FMDV in cattle. This needs to be confirmed by virus challenge experiments in cattle.

Because peptides alone are poorly immunogenic, in our study, in order to improve the immunogenicity of the

Table 2. Protection of guinea pigs immunized with fusion protein and DNA vaccines against challenge

Vaccine tested	Vaccine dose (μg/animal)		Protection ratio[a]
	First	Second	
Fusion protein of pAS-P	400	400	6/7 (86 %)
pWR590 (control)	400	400	0/5 (0 %)
pAS1-E	200	200	3/5 (60 %)
pAS1-EΔCpG-ODN	200	200	3/7 (43 %)
pHook-2 (control)	200	200	0/5 (0 %)

[a]Protection was defined as the absence of FMD symptoms for a period of 7 days after FMDV challenge and expressed as the protection ratio (the ratio of the No. of protected animals and the No. of challenged animals). Guinea pigs were grouped as described in Table 1.

epitopes, the β-galactosidase molecule was chosen as the carrier of the epitopes. Carriers can improve the immunogenicity of epitopes because of their ability to provide the T-helper response (Francis *et al.*, 1987; Broekhuijsen *et al.*, 1986). In addition, β-galactosidase has a long life span in *E. coli* and fusion with β-galactosidase can extend the half-life of a short-lived molecule. Therefore, a β-galactosidase carrier may overcome the problem of rapid degradation of foreign tandem repeat 133–158~20–34~133–158 epitopes and lead to a continuous exposure to the host, thus enhancing the immunogenicity of the epitopes in the host. Guinea pigs immunized with the recombinant fusion protein of pAS1-P showed both neutralizing antibody response and T cell proliferation response. The FMDV challenge experiments showed that it could protect 86% of guinea pigs against the FMDV type Asia1 challenge.

Genetic immunization with naked DNA has been shown to induce long-lived humoral and cellular immunity in a variety of animal models, suggesting an enormous potential of applying this strategy to vaccination (Guranathan *et al.*, 2000). Studies on DNA vaccines against FMDV based upon DNA encoding virus genome, DNA expressing virus capsid proteins or a gene sequence encoding VP1 fusion protein have been reported (Chinsangaram *et al.*, 1998; Beard *et al.*, 1999; Huang *et al.,* 1999). However, the DNA-vaccinated animals were either partially protected or not protected at all from virus challenge, suggesting the need for improvement.

In eukaryotic mRNA, the occurrence of the consensus sequence GCC(A/G)CCATGG is well established as Kozak's consensus sequence in vertebrates including *Homo sapiens* (Kozak, 1987). Two nucleotide positions, the purine 3 bp upstream of the ATG codon and the G 1 bp downstream of the ATG codon, are particularly well conserved. It has been shown that mutation at either or both of these key nucleotide positions lead to a reduction of translation or to an increase in the ribosome's leaky scanning (Kozak 1987, 1997). Thus the Kozak consensus sequence is thought to be a favorable sequence for the ribosome to recognize the initiation ATG codon. Insertion of the Kozak consensus initiation codon upstream of the signal sequence of murine Igκ chain (Kozak 1987; Manthorpe *et al.*, 1993) in the vectors pAS1-E and pAS1-EΔCpG-ODN will increase synthesis of the secreted epitope protein. A sufficient amount of the antigen is helpful for increasing immune responses.

Several reports indicate that the magnitude of immune response is influenced by the ability of the expressed antigen to be secreted (Rice *et al.*, 1999; Qiu *et al.*, 2000). In contrast to intracellularly retained antigens, which are presented primarily by the MHC class I pathway, secreted antigens are presented to T cells by transfected cells as well as by professional antigen-presenting cell (APC). The form in which the antigen is secreted may influence the type of APCs involved in T-cell priming leading to presentation of an antigen to a Th1 or Th2 subset of T helper cells. In the case of DNA immunization, the ability of the antigen to induce a Th1 or Th2 response depends on the route of DNA administration as well as on the form in which the secreted antigen is presented to APCs (Lewis *et al.*, 1997). In this study, we constructed the vectors pAS1-E and pAS1-EΔCpG-ODN in which the heterogenous secretory signal

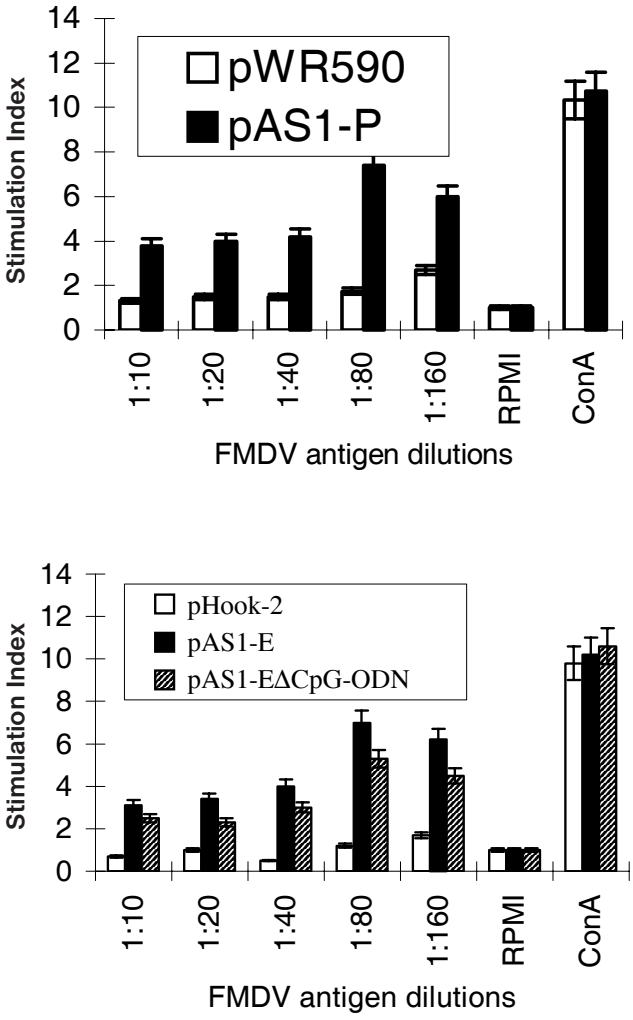

Fig. 3
T cell proliferation resulting from viral protein stimulation in guinea pigs immunized with fusion protein of vector pAS1-P (a) and with DNA vaccines pAS1-E and pAS1-EΔCpG-ODN (b)

T cells were stimulated with purified viral protein. The proliferation effect was determined by comparing the [^{3}H] thymidine taken up by the cells incubated with and without viral protein. Stimulation index is the ratio of mean cpm of culture stimulated with viral protein and mean cpm of culture not stimulated with viral protein. Guinea pigs were grouped as described in Table 1. Error bars represent SD. RPM = RPM medium.

sequence of murine Igę chain was fused in-frame to the tandem repeat 133–158~20–34~133–158 epitopes. The use of a secretory leader sequence of the murine Igκ chain may make the antigen protein secret out many types of cells and facilitate induction of host cell and antibody responses following DNA immunization. Some DNA adjuvants such as CpG-ODN have been shown to induce Th1 responses as characterized by the secretion of interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12) and IgG2a (Chu *et al.*, 1997; Roman *et al*., 1997). This kind of DNA adjuvant may thus offer significant advantages in supplementing the deficiency of most peptide and subunit vaccines that elicit mainly Th2 responses. It was recently shown (Chuang *et al.*, 2002) that the toll-like receptor 9 (TLR9) participates in recognition of CpG motifs. The motifs are internalized into acidified endosomal vesicles and released to trigger immune stimulation through a signaling cascade that involves the up-regulation of NF-κB (An *et al.,* 2002). APCs triggered by CpG ODN up-regulate expression of co-receptor molecules and secrete a variety of cytokines, including IL-12, IL-6, IL-1 and TNF-α.

In this study, we demonstrated that the vector pAS1-E containing multiple CpG motifs boosted the cellular and humoral immune responses to the vaccine-encoded antigen at different levels. We observed that guinea pigs treated with pAS1-E in comparison with pAS1-EΔCpG-ODN produced higher titers of neutralizing antibodies. T cell proliferation in the guinea pigs immunized with pAS1-E in comparison with pAS1-EΔCpG-ODN was slightly stronger. The virus challenge experiments showed that the protection ability of pAS1-E in comparison with pAS1-EΔCpG-ODN was stronger. Sixty % (3/5) and 43% (3/7) of the guinea pigs vaccinated with pAS1-E and pAS1-EΔCpG-ODN were protected from viral infection, respectively. Thus the advantage of adding CpG ODN to the corresponding vector was clearly demonstrated.

Although some efforts were exerted, the tested DNA vaccines protected guinea pigs from the FMDV type Asia 1 challenge not satisfactorily. The low protection may have many reasons concerning antigen protein expression, antigen secretion, antigen presentation, antigen degradation, etc.

At present, vectors encoding two VP1 epitopes (aa 141–160 and 200–213) and a host immunoglobulin molecule were constructed to produce a new type of FMD DNA vaccine. The vector pOS1 encoding only FMDV type O two VP1 epitopes (aa 141–160 and 200–213) displayed only a limited protection (33%). In an earlier study Wong *et al.* (2000) reported that the vectors pCEIM and pCEIS encoding FMDV type O two VP1 epitopes (aa 141–160 and 200–213) and a mouse or swine IgG molecule, respectively, elicited both FMDV-specific T-cell proliferation and neutralizing antibodies. In challenge experiments, three of three swines immunized with pCEIS were fully protected from FMDV type O challenge These results showed that fusion of VP1 epitopes with the carrier IgG could enhance protection from FMDV type O. Thus in our study, one of the reasons why the DNA vaccines elicited a low neutralizing antibody response and a lower protection may be lack of a suitable carrier.

In conclusion, we report here development of the recombinant fusion protein of pAS1-P and the pAS1-E, pAS1-EΔCpG-ODN DNA vaccines against FMDV type Asia1 using B and T cell epitopes. Guinea pigs immunized with these vaccines showed both neutralizing antibody and T cell proliferation responses. FMDV challenge experiments demonstrated for the first time that the recombinant fusion protein of pAS1-P and pAS1-E and pAS1-EΔCpG-ODN vaccines protected 86%, 60% and 43% of guinea pigs from FMDV type Asia 1 challenge, respectively. The results also indicated that the immune response of animals treated with the vector pAS1-E could be augmented by CpG ODN. Our results imply that the recombinant fusion protein vaccine of pAS1-P may serve as a candidate of a vaccine against FMDV type Asia 1 in cattle.

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