NOTES

CpG DNA enhances the immune responses elicited by the DNA vaccine against foot-and-mouth disease virus in guinea pigs

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Abstract **CpG DNA is DNA sequence that has immune stimulatory effects. Several lines of investigation over the past few years indicate that CpG DNA plays an important role in the induction of immune responses to DNA vaccines. In this study, CpG DNA-containing synthetic oligodeoxynucleotide (CpG-ODN) was cloned into the eukaryotic expression plasmid encoding a fusion protein containing β- galactosidase from *E. coli* and immunogenic epitopes of foot-and-mouth disease virus (FMDV) type O, and the immune responses induced by the plasmid were assayed. The results showed that guinea pigs immunized with the recombinant plasmid containing CpG-ODN generated a higher level of FMDV-neutralizing antibody and a stronger T cell proliferative response and protection against viral challenge than those receiving the plasmid containing no CpG-ODN. Our study demonstrated that it is an effective route to enhance the efficacy of DNA vaccines by inserting exogenous CpG DNA into the plasmids, and the DNA vaccine developed here is a promising candidate to prevent FMDV infection.**

Keywords: CpG DNA, DNA vaccine, foot-and-mouth disease virus, immnostimulatory sequence.

The immune stimulatory effect is another important function of DNA besides its function as the genetic code. The DNA with such a special effect is usually short and noncoding sequence which is known as CpG DNA(CpG motif) or CpG-immunostimulatory sequence. These sequences are particular hexamers in which an unmethylated CpG dinucleotide is preceded on the 5′ side by two purines and followed on the 3′ side by two pyrimidines[1,2].

CpG DNA is more frequent in the genomes of bacteria, viruses and nonvertebrates than of vertebrates, and the cytosines in CpG dinucleotides are usually largely methylated in vertebrates. *In vitro* experiments indicated that CpG motifs in bacterial DNA or synthetic oligodeoxynucleotide containing CpG motifs (CpG-ODN) can trigger direct B-cell activation with T-cell-independence and induce B cells to secrete immunoglobulin and interleukin 6 (IL-6)[2]. In addition to its direct effects on B cells, CpG DNA also directly activates monocytes, macrophages and dendritic cells to upregulate their expression of co-stimulatory molecules and to secrete a variety of Th1 cytokines including IL-12, tumor necrosis factor α (TNF-α) and interferon α (IFN-α) which in turn activate natural killer cells[3,4].

With the unfolding of the mechanisms of DNA vaccination, it has been recognized that CpG DNA plays an important role in the induction of immune responses to DNA vaccines[5,6]. Generally, the plasmids used as DNA vaccines contain certain number of CpG motifs. It is these CpG motifs that act as an intrinsic adjuvant to enable minute amount of antigen expressed by recombinant plasmid to induce strong immune responses. So DNA vaccines may be considered to have two essential components, one of which is the transcription unit comprising a promotor, antigen cDNA and poly-adenylation addition sequence which together direct protein synthesis, and the other is the immunoregulating unit containing CpG motifs which improves generation of antigen-specific immune responses[7]. Thus, it may be possible to enhance the efficacy of DNA vaccines by adding additional CpG motifs to them. However, previous strategies to investigate the immunostimulatory effects of CpG DNA on DNA vaccines are only involved in coadministration of exogenous CpG DNA with DNA vaccines. Here we designed and synthesized a CpG-ODN and then cloned it into eukaryotic expression plasmid encoding a fusion protein containing β-galactosidase (β-gal) from *E. coli* and immunogenic epitopes of foot-and-mouth disease virus (FMDV) type O to evaluate further its effects on the immune responses in gene-vaccinated guinea pigs.

1 Materials and methods

(i) Plasmids and host strains of *E. coli*. Plasmid pBS-SH contains the entire swine immunoglobulin G (IgG) heavy chain gene[8]. Plasmid pWR590FZ contains a fusion gene coding for β-gal and FMDV antigen peptide which consists of residues of amino acids 21—40, an important T cell epitope, and 141—160, a major B cell epitope, of type O FMDV VP1 protein. To improve their immunogenicity, these two peptide epitopes were ligated into a tandem repeat 141—160(20AA)–21—40 (20AA) –141—160 (20AA). Both plasmid pBS-SH and plasmid pWR590FZ are kept in this laboratory. The eukaryotic expression vector pCDM8 and host strain of *E. coli* Top 10 are kept in Department of Biology, Hong Kong University of Science and Technology.

(ii) PCR. The signal sequence of swine IgG heavy

chain gene was amplified from plasmid pBS-SH. The sense primer with a *Xho* I site on its 5′ side and the antisense primer with a *Nde* I site on its 5′ side were 5′-TG ATG CTC GAG ATA TGG AGT TTC GG-3′ and 5′-AGG CCT CCC ATA TGC TCC ACC AGC TTC TCC- 3′ respectively. The amplification protocol consisted of an initial denaturation cycle of 5 min at 94℃, followed by 30 cycles of 30 s at 94℃, 30 s at 55℃ and 30 s at 72℃, then by a final step of 5 min extension at 72℃. The fusion gene coding for β-gal and FMDV antigen peptide was amplified from pWR590FZ. The sense primer flanked by a *Nde* I site on its 5′ side and the antisense primer flanked by a *Not* I site on its 5′ side were 5′-GGA AAC ACA TAT GAC CAT GAT TAC GG-3′ and 5′-TAA GTA GCG GCC GCCT GCA GCT ATT AGG-3′respectively. The same reaction protocol as above was used except that the extension time was 90 s.

(iii) CpG-ODN. The CpG-ODN used herein was designed according to the previous reports[2,5,6]. The sense strand is 5′-GATCCAACGTTCCTGACGTTCCTGACG-TTCCCGGGACGTTCCTGACGTTG-3′, and the antisense strand is 5′-GATCCAACGTCAGGAACGTCCC-GGGAACGTCAGGAACGTCAGGAACGTTG-3′. CpG dinucleotides are underlined. After two strands were chemically synthesized, they were annealed to form a double-stranded fragment.

(iv) Animal vaccination. The plasmid was isolated and purified using Wizard DNA Purification System (Promega) and was dissolved in 0.9% NaCl at a concentration of 1 mg/mL. Guinea pigs weighting 150—200 g were inoculated intramuscularly in rear legs with 0.2 mL of plasmid DNA at 3—4 sites and was boosted 3 weeks later. The negative control guinea pigs received the same amount of plasmid DNA without any inserts (pCDM8).

(v) Neutralizing antibody assay. Two-day-old suckling mice were inoculated intraperitoneally with 100 μL of undiluted antisera obtained from vaccinated guinea pigs. After 20 h, all mice were challenged with serial dilutions of type O FMDV to determine the median lethal dose (LD_{50}). Neutralizing index (NI) which represents the titer of neutralizing antibody is calculated as the following[9]: NI = – lg (experimental group LD_{50}/nagative control group LD_{50}).

(vi) T cell proliferation assay. The T cells were isolated from spleen of guinea pigs 4 weeks after the second inoculation and were cultured in triplicate using 96-well flat-bottom plates at 1×10^5 cells per well. The cells were stimulated with serial dilutions of pure type O FMDV antigen at 37℃ for 60 h followed by incubation with [^{3}H]-thymidine for 12 h. As a positive stimulator, 50 μg/mL of concanavalin A (Con A) was used. After the cells were harvested, the [^{3}H]-thymidine incorporation into DNA was measured. The results are expressed as counts per minute (cpm) and stimulation index (SI): SI = cpm with antigen/cpm without antigen. SI≥2 is regarded as a positive response.

(vii) Viral challenge assay in guinea pigs. The guinea pigs were challenged with 0.2 mL of viral solution containing 50 guinea pig infectious dose ($50ID_{50}$) type O FMDV (strain of Hongkong/1997) at week 7 after the second vaccination. FMDV solution was delivered by both intradermal inoculation into each rear leg and coating on the surface of skin that had been scraped intentionally at metatarsus. The guinea pigs were observed for 2 weeks following challenge for signs of infection.

2 Results

(i) Construction of recombinant plasmids used for vaccination. Two amplified fragments, the signal sequence of swine IgG gene and the fusion gene coding for β-gal and FMDV antigen peptide, were digested with the corresponding restriction endonucleases and were then ligated with vector pCDM8 that had been digested with *Xho* I and *Not* I to form the construct pCDM8FZ1. Synthesized CpG-ODN was inserted into pCDM8FZ1 at *Bam* H I preceded with the poly-adenylation sequence to produce a plasmid containing exogenous CpG motifs designated pCDM8FZ2 (fig. 1). The recombinant plasmids were confirmed by digestion of restriction endonucleases, PCR and sequence analysis using a DNA automatic sequencer.

(ii) Neutralizing antibody responses. At week 2 and 4 after the second inoculation, both pCDM8-FZ1 and pCDM8-FZ2 elicited FMDV-neutralizing antibody responses, but the higher neutralizing index in pCDM8- FZ2 group implied that the response to pCDM8FZ2 is stronger than that to pCDM8-FZ1 (table 1).

Table 1 Neutralizing index in guinea pigs ($x \pm$ SE)

Test sample	Number of animals	Week post secondary inoculation	
		2	4
pCDM8FZ1	6	0.50±0.04	1.15 ± 0.13
pCDM8FZ2	6	1.40±0.15*	1.74± 0.16**
pCDM8	6	0	0

* and ** indicate significant differences from pCDM8FZ1 (*P < 0.01, **P < 0.05, by ANOVA).

(iii) T cell proliferative response. As shown in table 2, the spleen T cells of pCDM8-FZ2-vaccinated guinea pigs exhibited proliferative responses when exposed to all five serial dilutions of pure FMDV antigen. The peak of proliferation was observed at dilutions of 1∶50 and 1∶100. But the T cells from pCDM8-FZ1-vaccinated guinea pigs showed proliferative responses

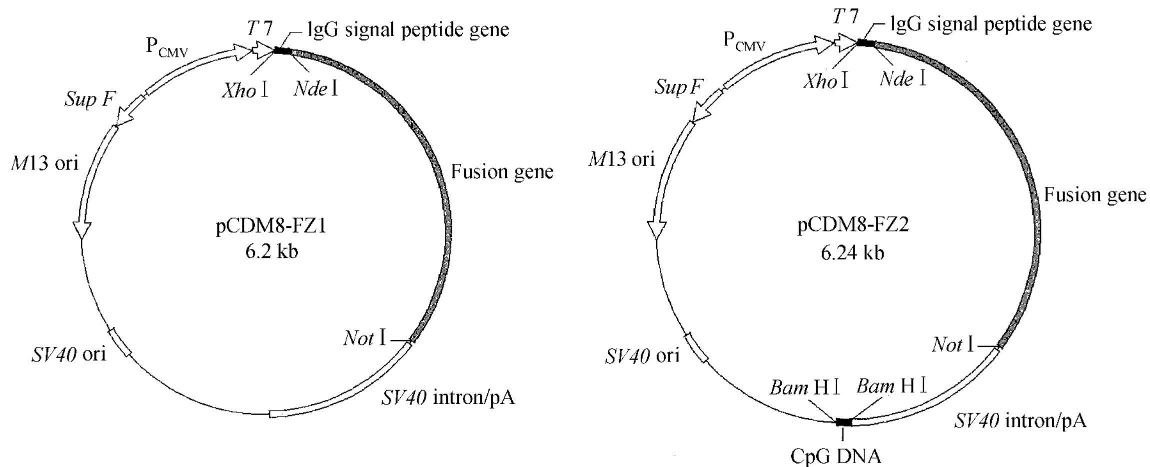

Fig. 1. Construction of plasmid pCDM8-FZ1 and pCDM8-FZ2. Fusion gene: gene encoding β-gal and FMDV antigen peptide.

Table 2 Proliferation of guinea pig spleen T cells ($x \pm SE$)a)

Test sample	FMDV antigen dilution					Con A
	1 ∶ 50	1 ∶ 100	1 ∶ 200	1 ∶ 400	1 ∶ 800	
pCDM8-FZ1	3.92±0.34	3.25±0.41	2.69±0.34	2.33±0.31	2.51±0.24	7. 33±1.43
	2.50±0.41**	2.07±0.35	1.71±0.48	1.48±0.20	1.60±0.18	4.66±1.40
pCDM8-FZ2	9.01±0.85*	8.09±1.22	3.72±0.22	4.93±0.61	3.48±0.40	5.94±0.63
	5.75±0.54**	5.15±1.11	2.37±0.70	3.14±0.94	2.21±0.61	3.78±0.56
pCDM8	1.38±0.21*	1.30±0.16	1.56±0.19	2.10±0.25	2.09±0.17	6.21±1.20
	1.05±0.19**	0.82±0.12	0.99±0.25	1.34±0.14	1.29±0.14	3.95±1.10

a) Results are expressed as cpm ($\times 10^3$) (*) and SI (**). 6 guinea pigs were tested per group.

only when stimulated with 1 ∶ 50 and 1 ∶ 100 dilutions of viral antigen, and the responses are much less stronger than those of pCDM8-FZ2 group ($P < 0.01$, by ANOVA).

(iv) Protection of guinea pigs against viral challenge.

PCDM8-FZ2 elicited a stronger neutralizing antibody response and T cell mproliferative response than pCDM8-FZ1. We next sought to determine whether the immunity generated in pCDM8-FZ2-vaccinated guinea pigs afforded them stronger protection against a FMD viral challenge. Table 3 reveals that the guinea pigs receiving pCDM8-FZ2 exhibit a stronger ability to prevent viral infection than those receiving pCDM8-FZ1(P<0.01, by t test).

Table 3 Protection of guinea pigs against viral challengea)

Test sample	Number of challenged	Number of protected	Protection rate (%)
pCDM8-FZ1	6	2	33
pCDM8-FZ2	6	5	83
PCDM8	5	0	0

a) Male guinea pigs were used.

3 Discussion

The immunostimulatory effect of DNA was first reported by Tokunaga et al.[10]. They observed that a DNA-rich fraction extracted and purified from *mycobacterium bovis* BCG exhibited strong antitumor activity against various syngeneic tumors in mouse and guinea pig. A series of subsequent studies led to the conclusion that the antitumor activity of DNA was attributed to the lymphocyte activation caused by immunostimulatory sequences in particular base contexts presented in DNA known as CpG motifs[1]. Published evidence suggested that CpG DNA was required for DNA vaccines to induce effective immune responses. While testing expression vectors containing the lacZ gene, which encoding β-gal, for their efficiency in gene vaccination of mice, Sato et al.[5] observed that the reduction of CpG DNA in plasmid decreased markedly the humoral and cellular immune responses to β-gal. Subsequent experiment showed that coadministration of exogenous CpG DNA with plasmid encoding circumsporozoite protein (CSP) of *plasmodium yoelli* malaria significantly increased the production of anti-CSP in mice[6], which demonstrated that the efficiency of DNA vaccines can be improved by adding additional CpG DNA. In this work, a CpG-ODN was cloned into the recombinant expression plasmid encoding type O

FMDV antigen to evaluate further the effect of CpG DNA on immunogenicity of a DNA vaccine. The results that the DNA vaccine containing CpG-ODN induces stronger immune responses and protects guinea pigs against viral infection more effectively than that containing no CpG-ODN demonstrated that it is an efficient route to enhance the efficiency of a DNA vaccine by engineering exogenous CpG DNA into the plasmid.

It appears that CpG-ODN is more effective in promoting the proliferative T cell response than in improving the FMDV-neutralizing antibody response. This may be explained by the features of immune stimulation by CpG DNA. It has now become clear that CpG DNA serves as an intrinsic adjuvant for DNA vaccines[7]. CpG DNA can activate the dendritic cells and other antigen-presenting cells in the anatomic region to which the DNA vaccine is administered and increase the efficiency of antigen presentation, and thus elicit strong antigen specific immune responses, especially cellular immune responses[13]. It is also recognized that CpG DNA upregulate a Th1-biased cellular and humoral immune response and induces a Th1-like pattern of cytokine production such as IL-12, IL-18 and IFN-γ[3].

The cytomegalovirus (CMV) immediate early promoter is a strong promoter which is used frequently for transcription of the target gene in muscle cells. The fusion gene was under the control of CMV promoter in the recombinant plasmids used in this study, thus the antigen can be expressed efficiently. The inserted CpG-ODN is located in the backbone of the plasmid, and it has no influence on the expression of the fusion protein[7]. There are a number of CpG motifs, and their immune-enhancing efficiency is different in degree[2]. The CpG-ODN used herein contains one CpG motif 5′-AACGTT-3′ and four repeats of CpG motif 5′-GACGTT-3′, both of which have been proved to be strong immune enhancers[5,6]. Further studies are required to optimize the type and number of CpG motifs for enhancing DNA vaccine efficacy.

At present, vaccination is the main available way to prevent outbreaks and spreads of foot-and-mouth disease (FMD), a severe contagious disease of livestock caused by FMDV. DNA vaccines have emerged as a kind of exciting new vaccine that promises significant advantages over conventional vaccines. However, to date no promising DNA vaccines against FMDV have been reported yet. The fact that guinea pigs inoculated with the recombinant plasmid pCDM8-FZ2 constructed herein can be protected effectively against lethal infection suggests that this DNA vaccine is a promising vaccine candidate against FMDV.

The effort to investigate the effects of CpG DNA on immunogenicity of the DNA vaccine by cloning the CpG-ODN into the plasmid is beneficial for defining the mechanisms of gene immunization and the strategy to improve its efficiency. Besides its adjuvant effect on DNA vaccines, CpG DNA has also been found to enhance significantly immune responses to protein vaccines when used as an adjuvant[11]. Furthermore, CpG DNA exhibits a higher immune-enhancing activity than that of alum which is a conventional adjuvant. In addition, CpG DNA is also a highly effective vaccine adjuvant for tumor vaccination strategies[12]. Based on these findings, it could be concluded that CpG DNA is a new immune enhancer for vaccination applications with enormous future potential.

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