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Regulating Effects of Novel CpG Chitosan-nanoparticles on Immune Responses of Mice to Porcine Paratyphoid Vaccines

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Objective To study the regulating effects of a novel CpG oligodeoxynuleotide and the synergistic effect of chitosan-nanoparticles (CNP) with CpG on immune responses of mice, which were used to develop a novel immunoadjuvant to boost immune response to conventional vaccines. **Methods** A novel CpG ODN containing 11 CpG motifs was synthesized and its bioactivities to stimulate the proliferation of lymphocytes of pig *in vitro* were detected. Then it was entrapped with CNP prepared in our laboratory by the method of ionic cross linkage, and immunized Kunming mice were co-inoculated with paratyphoid vaccine. The peripheral blood was collected weekly from the tail vein of inoculated mice to detect the contents of IgG, IgA, IgM, and specific antibody against salmonella as well as the levels of interleukin-2 (IL2), IL-4, and IL-6 by SABC-ELISA assay. The numbers of leucocytes, monocytes, granuloytes, and lymphocytes were calculated separately using the routine method. The experimental mice were orally challenged with virulent salmonella 35 days after inoculation. **Results** This CpG ODN could remarkably provoke the proliferation of lymphocytes of pig *in vitro* in contrast with the control ($P<0.05$). Compared with those of the control, immunoglobulins, including IgG, IgA, IgM, and specific antibodies to paratyphoid vaccine, increased significantly in sera from the CpG or CpG-CNP-vaccinated mice ($P<0.05$). IL-2, IL-4, and IL-6 increased remarkably in sera from immunized mice ($P<0.05$). The leucocytes, monocytes, granuloytes, and lymphocytes of the mice immunized with CpG or CpG-CNP were also increased in number ($P<0.05$). After the challenge, these immunity values were elevated in the mice vaccinated with CpG or CpG-CNP. The immunized mice all survived, while the control mice fell ill with evident lesions with diffuse hemorrhage in stomach, small intestine, and peritoneum. **Conclusions** CpG ODN entrapped with CNP is a promising effective immunoadjuvant for vaccination, which promotes humoral and cellular immune responses, enhances immunity and resistance against salmonella by co-administration with paratyphoid vaccine.

Key words: CpG oligonucleotide; Mice; Immune responses; CNP; Paratyphoid vaccine

INTRODUCTION

CpG oligodeoxynucleotide is a newly emerging powerful adjuvant inducing a broad array of immune responses to a wide variety of antigens. Previous studies showed that CpG motifs activate B cells and dendritic cells (DC), trigger immune cascade including production of cytokines, chemokines, and IgM, and proinflammatory maturation/activation of professional antigen-presenting cells[1-2]. These characteristics enable CpG ODN to act as an immune adjuvant, accelerating antigen-specific immune responses[3]. Though there are studies on the effects of CpG motifs to a variety of vaccines on immune responses, no study on regulating effect of CpG motifs to paratyphoid vaccine is available.

Chitosan is a nontoxic and biodegradable polysaccharide that has recently emerged as a promising candidate for gene delivery[4]. It has the property of compatibility with organism and can be degraded by some enzymes *in vivo*. Owing to its unique poly-cation, it is a kind of potential slow-releasing material[5-6] and has a high potential for transferring DNA molecules[4,7]. Rudimental researches have made it clear that CNP system has a broad practice prospect in biomedical science. But up to now, how CpG motifs entrapped with CNP regulate animal immune responses still remains unknown.

In order to further explore the effect of CpG

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motifs and CpG-CNP on the regulation of immune response of mice, oligodeoxynuleotide containing eleven CpG motifs was synthesized, enwrapped with CNP, and then used to inoculate mice together with the paratyphoid vaccine. It is expected to investigate, for the first time, the adjuvant efficacy of the novel CpG ODN to augment the immune responses of mice co-administe with the paratyphoid vaccine and the synergy of CNP with CpG on immunity of mice, which would lay the rudimental basis to develop new immunopotentiator with high safety and long effect.

MATERIALS AND METHODS

Conventional Vaccine

The paratyphoid vaccine (provided by Chengdu Medicine and Appliances Factory, China) including attenuated salmonella was used in this work as a conventional vaccine to immunize swine in China. One dose per pig includes 2×10^9/mL attenuated salmonella bacteria.

Preparation of CPG ODN

Immunostimulatory oligodeoxynuleotide (ODN), synthesized by Shanghai Genebase Co., has 88 bases containing eleven CpG motifs: CGCTGCAGAACGTTGTCGTCAACGTTGTCGTC AAGCTTGACGTTATCGATGGCGTTGACGTTGA CGTCATCGATGTCGTTCTGCAGCG. According to CPG ODN, a pair of primers was synthesized as follows: sense primer, CGCTGCAGAACGTTGTC; anti-sense primer, CGCTGCAGAACGACATCG. Polymerase chain reaction (PCR) mixture (50 μL) contained 5 μL of 10×PCR buffer, 2.5 μL of 10 mmol/L dNTP, 10 pmol each of the 5′ and 3′ primers, 10 pmol CpG ODN, and 1 μL of Taq polymerase (5 units/μL). PCR was carried out at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 seconds, at 58℃ for 30 seconds, at 72℃ for 30 seconds, and then a final extension at 72℃ for 5 min. The size and yield of the amplified CpG fragment were determined by 1.5% agarose gel electrophoresis.

Proliferative Assay of Lymphocyte of Landrace

Landrace blood lymphocytes were isolated by lymphocyte separation medium (density, 1.077±0.002) in a bacteria-free condition, adjusted to 1×10^6 cells/mL, then cultured in RPMI 1640 complete medium (including 10% fetal bovine serum, 100 ug/mL benzylpenicillin, 100 ug/mL streptomycin) in a 5% CO_2 atmosphere at 37℃. The CpG ODN, paratyphoid vaccine or their mixture was added separately into the cells at the dose of 0.2 μg/mL to test their stimulatory effect on the immune cells. After cultivation for 72 h, 10 μL 5 mg/mL MTT (3, (4, 5-Dimethylthiazoyl-2-yl) -2, 5-diphenyltetrazolium bromide, Sigma) was added into the media. After 3 h, 50 μL HCl-SDS (10% SDS, 0.01 mol HCl) was added into the media. After 2 h, the cell culture plate was taken out of the incubation chamber and placed at room temperature for 20 min before the absorbance of A_{570} of the samples was determined with Bio-Rad Microplate Reader 3550.

Preparation and Detection for CNP

Chitosan was provided by Chengdu Organic Chemistry Institute of CAS in Sichuan Province. Its molecular weight is 150 KD, and deacetyl degree is over 95 percent. CNP entrapped CpG (CpG-CNP) was prepared using the method of ion crossing[8]. Its configuration was observed under transmission electronic microscope with a granule diameter. Dispersity and zeta electricity potential were detected using Zetasizer 3000 HS/IHPL instrument (Malvern Instruments Ltd, Malvern, UK).

CNP Gel Retardation Assay

The packing rate of CpG-CNP was detected by 1.5% agarose gel electrophoresis.

Animal Vaccination

Sixty 6-week-old female Kunming mice purchased from the Animal Center of Sichuan University were randomly divided into four groups (A, B, C, and D), fifteen mice per group. Group A was injected with naked CpG DNA (30 pmol per mouse), group B with CpG-CNP (6 pmol per mouse) in the left and right quadriceps, and group C with oral CpG-CNP (6 pmol per mouse). These three groups were co-administed with paratyphoid vaccine (one twentieth dose of pig for per mouse, 5×10^7/mL inactivated salmonella per capita). Group D was injected with only paratyphoid vaccine as control. After 35 days, the mice were challenged with virulent salmonella strains (provided by Veterinary Medicine Supervision Institute of China, Beijing), 50 μL blood was collected from the tail vein of each mouse on days 0, 7, 14, 21, 28, 35, 42, and 49 after immunization.

Immunological Assays Counting of Immune Cells of Mice

The immune cells of mice were counted by a routine method. Giemsa staining for blood smears was performed to sort out the number of neutrophils, monocytes, and lymphocytes.

Assay of IgG, IgM, and IgA

Ninty-six-well flat-bottomed plates (Nuclon,

Roskilde, Denmark) were coated with 100 μL of 10^5 (IgG) or 10^4 (IgA, IgM) fold diluted sera from immunized mice in bicarbonate coating buffer (30 mmol/L Na_2CO_3, 75 mmol/L $NaHCO_3$, pH9.6) at 4℃ overnight. The plates were washed three times with PBS (0.2 mol/L Na_2HPO_4, 0.2 mol/L NaH_2PO_4, pH7.2) containing 0.1% Tween-20, and then 100 μL rabbit anti-mouse IgG (Takara Company, Dalian, China) diluted 1:800 in 1% BSA buffer was added to each well. After incubation for 1 h at 37℃, the plates were washed three times again, and 100 μL 1:1000 sheep anti-rabbit IgG-ABC was added. After incubation for 1 h at 37℃, the plates were washed again and 100 μL TMB substrate (Sigma, St. Louis, MO, USA) in 0.1 mol/L phosphate-citrate buffer (pH 5.4) was added, and the plates were incubated at 37℃ for 30-60 min, before 50 μL 2 mol/L H_2SO_4 was added to stop the color development. The absorbance of the samples was read at 450 nm on Microplate Reader 3550 (Bio-Rad, Hercules, California, USA).

Assay of Specific Antibody of Immunized Mice

Ninty-six-well flat-bottomed plates (Nuclon, Demark) were coated with 100 μL antigen protein of salmonella (20 μg/mL, diluted in bicarbonate coating buffer) and incubated overnight at 4℃. Plates were washed three times in PBS Tween-20 washing buffer. Sera of immunized mice were properly diluted in 0.1 mol/L PBS containing 1% BSA, and added into plates, 100 μL per well. The plates were incubated for 1 h at 37℃. After washing five times, 100 μL sheep-anti-mouse IgG-ABC diluted 1:1200 in 1% BSA buffer was added to each well (containing 1% BSA), and the plates were incubated for 1 h at 37℃. After washing five times and addition of 100 μL SABC (diluted 1:1200), the plates were incubated for 1 h at 37℃. After washing five times and addition of 100 μL TMB substrate (Sigma, St. Louis, MO, USA) in 0.1 mol/L phosphate-citrate buffer (pH 5.4), the plates were incubated for 30-60 min at 37℃. The reaction was stopped with 2M H_2SO_4 (50 μL/well), and the absorbance of samples were read at OD_{450} on Microplate Reader 3550 (Bio-Rad, USA).

Assay of Interleukin-2, 4, and 6 in Immunized Mice

Ninety-six-well flat-bottomed plates (Nuclon, Roskilde, Denmark) were coated with 100 μL of 100-fold diluted sera from immunized mice in bicarbonate coating buffer (30 mmol/L Na_2CO_3, 70 mmol/L $NaHCO_3$, pH9.6) at 4℃ overnight. Rabbit-anti-mouse IL-2, IL-4, IL-6 (Bostar Biological Corp. in Wuhan, diluted 1:800 in 1% BSA buffer) were used as Ab_1, sheep-anti-rabbit IgG-ABC (diluted 1:800 in 1% BSA buffer) as Ab2 and TMB as substrate. The absorbance of the samples was read at 450 nm on Microplate Reader 3550 (Bio-Rad, Hercules, California, USA).

Immunoprotection Test

Thirty-five days after immunization, the mice were challenged by oral administration of virulent salmonella to test their resistance against infection. Two weeks after the challenge, all the surviving mice were sacrificed and autopsied for observation of pathological lesions.

Statistical Analysis

Data from all groups were statistically analyzed by Student's *t* test. $P<0.05$ was considered statistically significant.

RESULTS

Stimulatory Effect of CpG ODN on Lymphocytes of Landrace in vitro

The CpG and paratyphoid vaccine provoked remarkable proliferation of lymphocytes of pig *in vitro* in comparison with that of control group ($P<0.05$), and both had synergetic stimulating effect on the proliferation of porcine immune cells (Table 1).

TABLE 1

Proliferative Reaction of CpG ODN on Lymphocytes of Landrace

Group	OD_{570}
CpG	0.298±0.035 [b]
CpG+ Paratyphoid Vaccine	0.425±0.039 [a]
Paratyphoid Vaccine	0.206±0.029[b]
Control	0.055±0.015 [c]

Note. The data with different superscript letter are significantly different ($P<0.05$).

Preparation of Chitosan Nanoparticles

Most of CNP were spheroid under transmission electron microscope founded that (Fig.1). The analyses

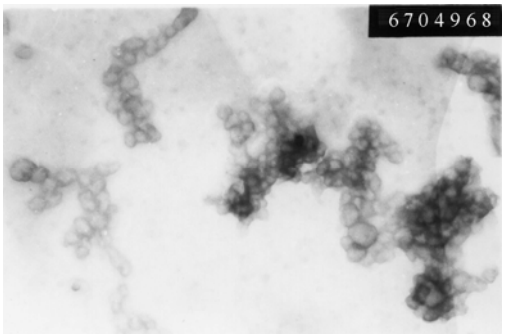

FIG. 1. Electronic micrograph of chitosan nanoparticle transmission (×50 000).

by Zetasizer 3000 HS/IHPL showed that the average granule diameter was 45 nm, multi-dispersity was 0.190 (Fig. 2), and zeta potential was +25.6 mV, suggesting that the CNP was positively charged.

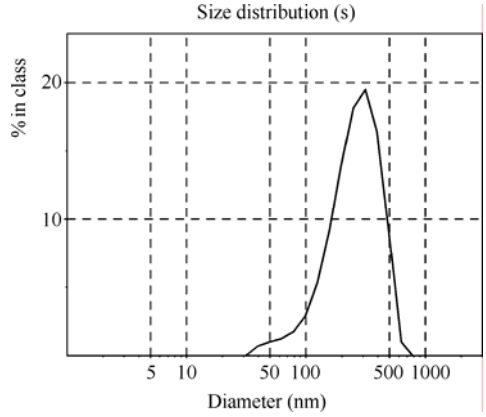

FIG. 2. Distribution of CpG-chitosan nanoparticle size.

Gel Retardation Assay

Figure 3 shows the result of 1.5% agarose gel electrophoresis of CpG-CNP. CpG-CNP did not move out of sample hole and was entirely blocked, indicating that CpG was entrapped with chitosan.

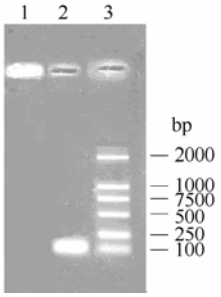

FIG. 3. Gel retardation assay of CpG-CNP (1.5% agarose electrophoresis). 1: CpG entrapped with CNP. 2: CpG. 3: DL2000 marker.

IgG, IgM, and IgA in Sera of Immunized Mice by ELISA

Figure 4 shows that IgG, IgM, and IgA increased in the sera of vaccinated mice of groups A, B, C, and were remarkably higher than those in group D. It could be elucidated that both naked CpG motifs and CPG enwrapped by CNP could stimulate the immunized mice to produce more IgG, IgM, and IgA (P<0.05).

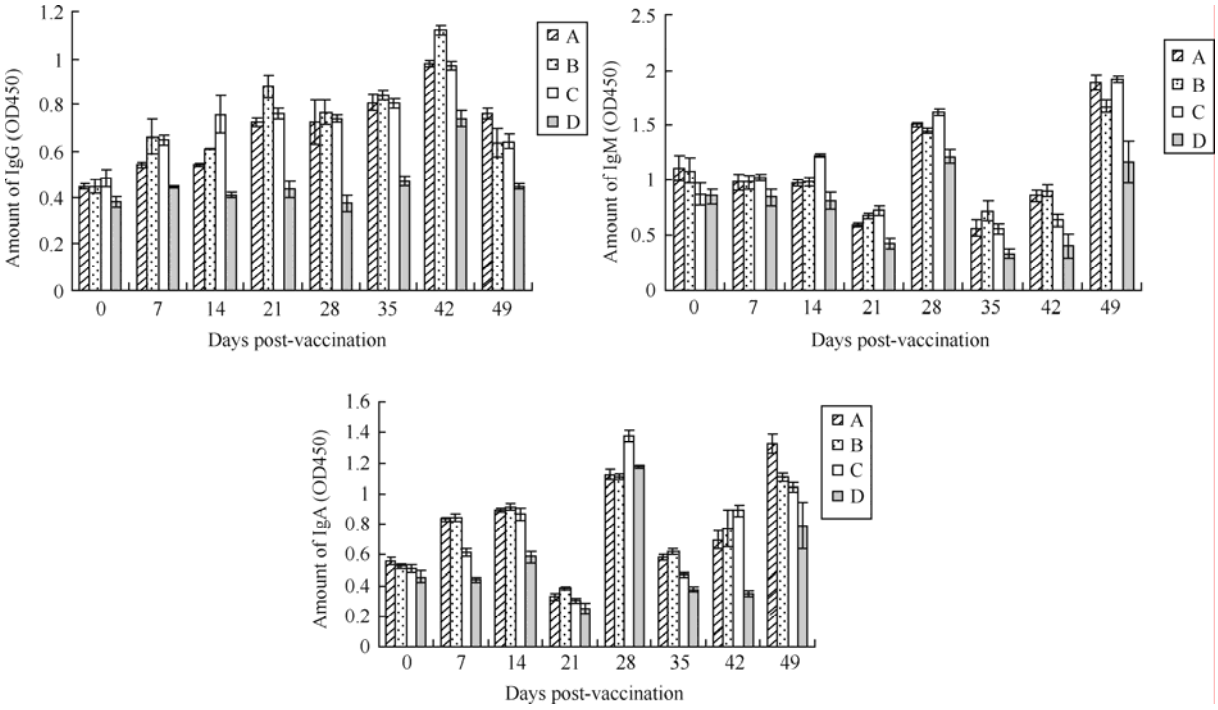

FIG. 4. Changes of IgG, IgM, and IgA contents in sera of experimental vaccinated mice.

Specific Antibody Responses to Vaccination in Mice

Seven days after immunization, the specific antibody could be detected, and the content of antibody of the mice co-immunized with CpG (including groups A, B, and C) rose significantly, compared with that of the control (group D) (P<0.05) (Fig. 5).

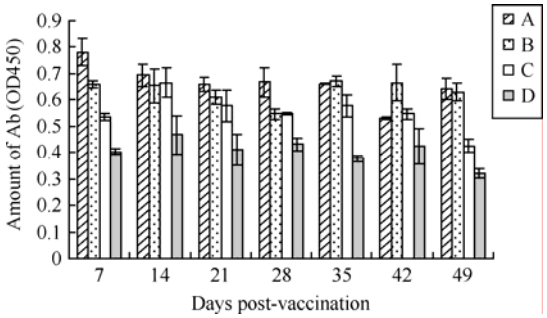

FIG. 5. Changes of content of specific antibody in immunized mice.

Assay of Interleukin in Immunized Mice

Compared with those of controls, IL-2, IL-4, and IL-6 increased significantly in mice co-immunized with naked CpG or CpG enwrapped with CNP ($P<0.05$) (Fig. 6). After the mice were challenged with virulent bacteria, IL-2, IL-4, and IL-6 in immunized groups were still remarkably higher than those in controls.

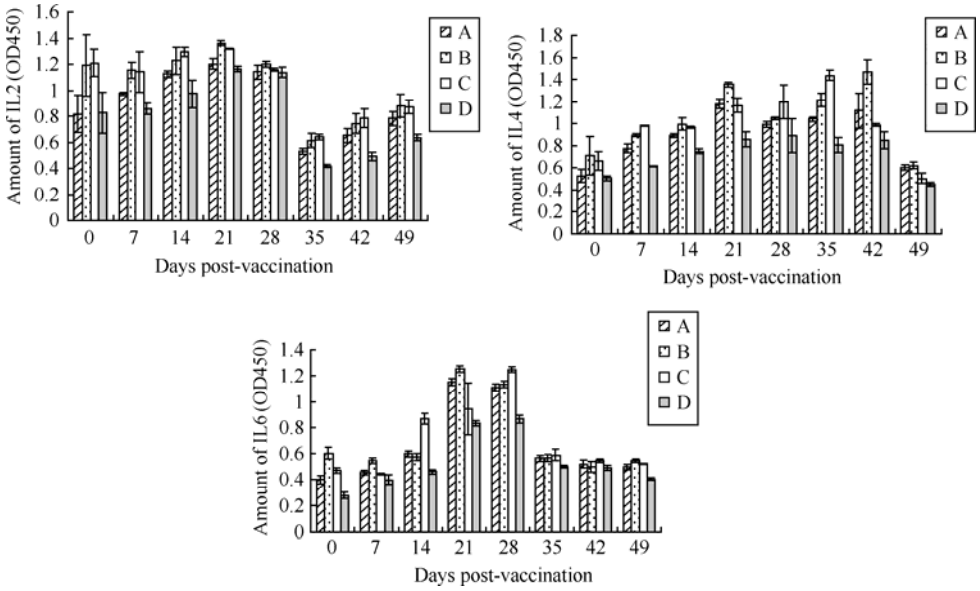

FIG. 6. Changes of IL-2, IL-4, and IL-6 levels in immunized mice.

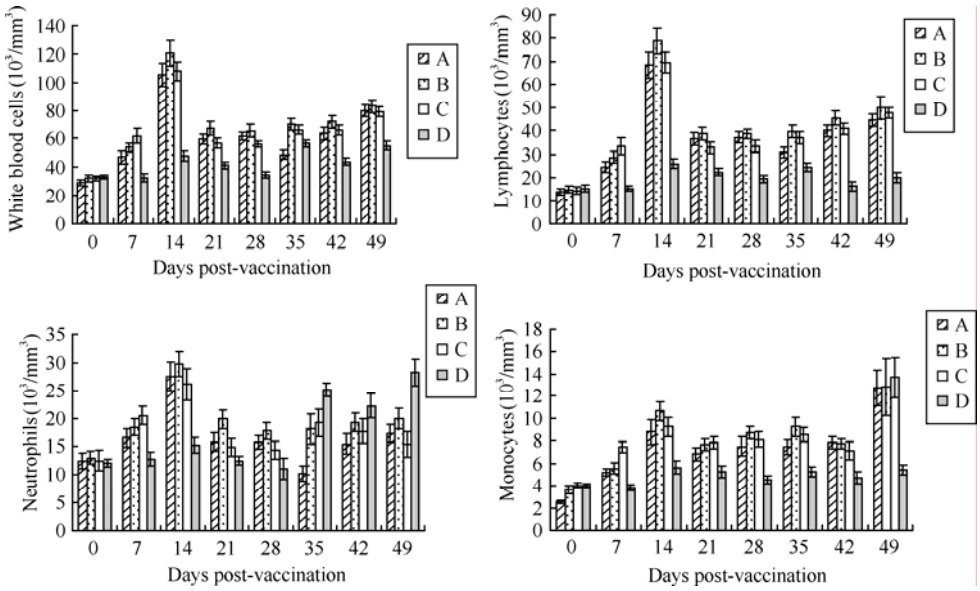

FIG. 7. Changes of numbers of leucocytes, monocytes, lymphocytes, and neutrophils.

Changes of the Number of Immune Cells

The results showed that the number of immunocytes in peripheral blood of mice co-immunized with CpG (including naked CpG and CpG enwrapped by CNP) had a remarkably increase in comparison with that in controls ($P<0.05$) (Fig. 7).

Challenge Results

Two weeks after oral administration of virulent salmonella, most of the mice inoculated with CpG (groups A, B, and C) survived with no abnormal symptoms, while the control group had evident symptoms of severe diarrhea. Autopsy of the mice exhibited, evident lesions, such as liver and spleen edema, stomach bleeding, and duodenum and jejunum catarrh in the control group.

DISCUSSION

Paratyphoid fever caused by salmonella is still a widespread disease[9-10] and a global health problem[11]. Paratyphoid vaccine plays an important role in preventing swine paratyphoid. However, the protective effect of the vaccine is frequently weakened in practical field due to continuous mutation of salmonella and immunosupression of animals by various severe stresses[12].

Previous studies have shown that CpG motifs activate a wide variety of innate immune responses and play a key role in gene immunization[13-14]. Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs induce B cells to proliferate and secrete IL-6, IL-10, immunoglobulin, and to express increased level of co-stimulatory molecules[15-16]. CpG motifs promote production of T-helper 1[17], induce maturation/ activation of professional antigen-presenting cells including macrophages, monocytes, and dendritic cells to produce cytokines, such as IL-12, IL-6, TNF-α, IFN-α, IFN-β, *etc*.[18-20], and promote natural killer (NK) cell lytic activity and gamma interferon (IFN-γ) secretion *in vivo* and *in vitro*[21-22]. CpG motif also enhances the expression of class II MHC and co-stimulatory molecules, such as B7-1 and B7-2, which also improve their capability of inducing B and T cell immune responses[23].

A novel CpG ODN synthesized in our laboratory containing eleven CpG motifs manifested a significant stimulatory effect on the proliferation of porcine lymphocytes *in vitro*, suggesting that it can be used to regulate porcine immunity. In this study, mouse model was used to study the immunoenhanceing effect of CpG ODN. Mouse models of salmonella infection have been used extensively to evaluate the feasibility of various vaccine attenuation strategies[24] and foreign antigen immunization[25]. In this study, CpG ODN enwrapped with CNP was employed to immunize the experimental mice together with paratyphoid vaccine to detect the immunological property. The results showed that specific antibody, IgG, IgA, IgM, IL-2, IL-4, IL-6 and the number of immune cells, all increased significantly in the mice co-administrated with CpG in contrast with those in the control group, suggesting that co-administration of CpG motifs with paratyphoid vaccine can significantly increase the level of immune responses in mice. The immune stimulatory effects of ODN containing unmethylated CpG dinucleotides depend on the flanking and the number of CpG motifs in oligonucleotides, and the spacing between individual CpG motifs[26-27]. CpG motifs flanked by two 5' purines and two 3' pyrimidines can exert optimal immune stimulatory effects[28]. Depending on different structure of individual motifs, the adjuvant effect of CpG ODN on DNA vaccines varies enormously and is usually specific for different animals[29-30]. Our synthetic CpG sequence backbone contains 88 bases including eleven specific CpG motifs, which are active to stimulate the cellular immunity *in vitro*. The results indicate that the CpG ODN can be used as an effective adjuvant of paratyphoid vaccine to increase the immunity of animals against infection.

It was reported that the molecular weight (MW), the deacetylation degree (DD) of chitosan, and the size of nanoparticles are the important factors affecting delivery efficiency[31-32]. As DD and MW of chitosan increase, the encapsulation efficiency increases while the release rate of material enwrapped into CNP decreases[33-34]. Besides, the common diameter of nanoparticles is over 100 nm, limiting its stability and penetration of cells *in vivo*. In our study, by modifying of the ratio of plasmids and chitosan, the diameter of CNP was relatively even. Transmission electron microscopy and nanoparticles granularity analysis showed that CNP was successfully prepared and molecular package of CpG was completed. Although the dosage of CpG in CNP enwrapped group was only one-fifth of CpG in non-enwrapped group, the immunological assay showed that CpG-CNP could obtain similar immunoadjuvant effects like naked CpG, which could also significantly raise the cellular and humoral immune level and resistance of mice against salmonella infection *in vivo*, suggesting that CNP can remarkably improve the immunostimulative

efficiency of CpG, and CpG-CNP can be used as a potential immunoadjuvant for vaccines.

Since pH in the stomach, harsh enzymatic environment in the gastrointestinal tract, and poor permeability of both genes and gene carriers across the intestinal epithelium can lead to disruption of DNA, oral CpG CNP has a poor efficacy. In order to solve this problem, we attempted to enwrap CpG with CNP as oral preparation. The experiment result showed that oral feeding of chitosan CpG nanoparticles could remarkably raise immune responses of mice, indicating that chitosan CpG nanoparticles possess characteristics for oral vaccination and can be used for the development of effective and economical immunopotentiator.

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