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A Novel Chitosan CpG Nanoparticle Regulates Cellular and Humoral Immunity of Mice

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Objective To develop a safe and novel immunoadjuvant to enhance the immunity and resistance of animals against *E. coli* infection. **Methods** An 88-base immunostimulatory oligodeoxynuleotide containing eleven CpG motifs (CpG ODN) was synthesized and amplified by PCR. The chitosan nanoparticle (CNP) was prepared by ion linking method to entrap the CpG ODN that significantly promotes the proliferation of lymphocytes of pig *in vitro*. Then the CpG- CNP was inoculated into 21-day old Kunming mice, which were orally challenged with virulent K88/K99 *E. Coli* 35 days after inoculation. Blood was collected from the tail vein of mice on days 0, 7, 14, 21, 28, 35, 42, and 49 after inoculation to detect the changes and content of immunoglobulins, cytokines and immune cells by ELISA, such as IgG, IgA, IgM, IL-2, IL-4, and IL-6. **Results** The CpG provoked remarkable proliferation of lymphocytes of pig *in vitro* in comparison with that of control group ($P<0.05$). The inoculation with CpG-CNP significantly raised the content of IgG, IgM, and IgA in the sera of immunized mice ($P<0.05$). The levels of IL-2, IL-4, and IL-6 in the mice significantly increased in comparison with those in controls ($P<0.05$), so was the number of white blood cells and lymphocytes in immunized mice. The humoral and cellular immunities were significantly enhanced in immunized mice, which resisted the infection of *E. coli* and survived, while the control mice manifested evident symptoms and lesions of infection. **Conclusions** CpG-CNP can significantly promote cellular and humoral immunity and resistance of mice against *E. coli* infection, and can be utilized as an effective adjuvant to improve the immunoprotection and resistance of porcine against infectious disease.

Key words: CpG motifs; Chitosan nanoparticles; Immune responses; Mouse; *E. coli*

INTRODUCTION

Immunoadjuvant plays a vital role in eliciting excellent immune responses to the inactivated, synthetic peptide and recombinant protein vaccines. Without the help of adjuvant, the immunogenicity of the vaccines is usually not enough to establish powerful immunoprotection against infectious pathogens[1]. Therefore, various adjuvants have been tried in the control of infectious diseases, especially in the development of novel recombinant genetic and DNA vaccines[2]. Generally, there are three main types of adjuvants being used in practice. The first is chemical molecules, bacteria and plant or components derived from them. They are still extensively utilized in current vaccinations. But their shortcomings are obvious, as they usually cause local tissue injury, and are not always successful in inducing a strong immune response[3]. The second is the cytokines, such as monokines, interleukins or interferons. As natural immune regulatory molecules, they have some remarkable advantages, including safety, specificity and efficacy. Their main disadvantages are that it is expensive to produce and purify them with a short-spanned bioactivity *in vivo*. The third is specific nucleic acids, such as the genes of cytokines and stimulatory DNA sequences, which are recently employed to promote the immunity of animals and humans to obtain satisfactory resistance against various infections. Among them, CpG sequences are currently used to induce strong immune responses to some vaccines characterized by the unmethylated CG dinucleotides within specific deoxynucleotide sequence contexts (CpG motifs) and their immunostimulatory bioactivity raises the immunity of vertebrate animals[4]. The CpG motifs are currently used to enhance the immune response in monkeys and mice[5-6].

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Chitosan is derived from chitin by deacetylation in the presence of alkali, and is well compatible with animal organism, which can be safely degraded by enzymes and cells *in vivo* and the degradation products are harmless[7]. Moreover, previous studies suggest that it is a kind of potential slow-relaying material, owing to its characteristics of unique poly-cation[8-9].

Recently, chitosan-nanoparticles（CNP） have been used in gene transfection and expression. Some researches indicate that chitosan could pack and condense DNA, forming small and dispersed particles, and CNP-DNA carries objective molecules into target cells[10-12]. Because the material release rate into enwrapped CNP is decided by chitosan's biodegradation and corrosion, the DNA enwrapped into CNP may be gradually released and lasted for a relative long period *in vivo*[13-14]. Besides, chitosan could also be utilized as a food additive and a medicine absorption enhancement agent[15-17].

Up to now, reports about the immunoadjuvant activity of CpG motifs and chitosan nanoparticles on animals *in vivo* are few. In order to explore safe novel efficacious adjuvants to enhance the immunity of animals against infectious diseases, the experiment was conducted to investigate the effect of special CpG oligodeoxynucleotides containing eleven CpG motifs enwrapped with chitosan nanoparticles on provoking the immunity of animals against challenge infections.

MATERIALS AND METHODS

Oligodeoxynuleotide CpG

Immunostimulatory oligodeoxynuleotides synthesized by Shanghai Sangon Co., have 88 bases containing eleven CpG motifs：5'> CGAGATCTAAC GTTGTCGTCGACGTCGTCGTCAGGCCTGACGT TATCGATGGCGTTGTCGTCAACGTTGTCGTTA ACGTTAGATCTCG< 3'. PCR primers for the ISS sequences are P1 5'>CGAGATCTAACGTTGTC<3', and P2 5'> CGAGATCTAACGTTAAC<3'. The sequence contains the Stu I site used for identification.

Preparation and Detection for CNP

Chitosan was provided by Chengdu Organic Chemistry Institute of Chinese Academy of Sciences (Sichuan Province, China). CNP was prepared as previously described[18], and then observed under transmission electronic microscope. The granule diameter, dispersivity and Zeta electronic potential of CNP were detected with Zetasizer3000HS/IHPL instrument(Malvern Instruments Ltd, Malvern, UK).

CNP Gel Retardation Assay

The packing rate of plasmids was detected by electrophoresis on 1.5% agarose gel.

Proliferation Assay of Lymphocytes of Pig

Pig 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 at a 24 cell culture plate in RPMI1640 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 was added respectively into the cells at the dose of 0.2 ug/mL to test their stimulatory effect on the immune cells. After incubation for 72 hours, 10 uL 5 mg/mL MTT (3, (4, 5-dimethylthiazoyl-2-yl)-2,5-diphenyltetra-zolium bromide, Sigma) was added into the media; after 3 hours, 50 uL HCl-SDS (10% SDS, 0.01 mol HCl) was added into the media for 2 hours. 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 BioRad Microplate Reader 3550.

Preparation of CpG ODN

In one tube, 79 μL water, 8 μL 10 mmol/L dNTP, 2 μL primers, 1 μL CpG template, and 10 μL 10× buffer were mixed and reacted in a PCR instrument (Thermo Hybaid P×2, Ashford, Middlesex, UK) with 30 cycles of amplification at 94℃ for 45 s, at 46.5℃ for 45 s, and at 72℃ for 30 s. This was followed by a final extension for 10min at 72℃. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination.

Animal Vaccination

Six-week female Kunming mice were divided into four groups, fifteen mice per group. Experimental mice were intramuscularly vaccinated in the left and right quadriceps respectively with 30 pmol naked CpG DNA and 6pmol CpG enwrapped with chitosan nanoparticles (Groups A1 and A2). Group B was orally fed with 30 pmol CpG enwrapped with chitosan nanoparticles. Group C was only injected with saline as the control group. Fifty μL blood was collected from the tail vein of mice on days 0, 7, 14, 21, 28, 35, 42, and 49 after immunization.

Calculation of Immune Cells of Mice

The immune cells of mice were counted. The blood smears were stained with Giemsa stain to sort out the number of white blood cells, neutrophils, monocytes, and lymphocytes.

Amount of IgG, IgA, and IgM

Ninety-six well flat-bottomed plates (Nuclon, Roskilde, Denmark) were coated with 100 μL sample sera from mice diluted in bicarbonate coating buffer (15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$, pH 9.6) at 4℃ overnight. Sheep anti-mouse IgG, IgA and IgM antibodies (Takara Company, Dalian, China) were used as the first antibody, SPA-HRP as the second antibody and TMB as substrate (Sigma, ST. Louis, MO, USA). Then OD450 of the samples was measured with a microplate reader 3550 (Bio-Rad, USA).

Content of IL-2, IL-4, and IL-6

The sera from mice diluted in bicarbonate coating buffer(15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$, pH 9.6) were coated into the 96 well flat bottom plates (Costar). Rabbit-anti-mouse IL2, IL4, and IL6 IgG (Bostar biological Co. in Wuhan) were used as Ab_1 and TMB as substrate. The content of IL2, IL4, and IL6 in immunized mice was measured with the method of SABC.

Test of Resistance Against Challenge

After inoculation for thirty-five days, the mice were orally fed with virulent *E. coli* strain at the dose of 0.2 mL, 2×10^9/mL bacteria, and observed for symptoms and lesions. Two weeks post-challenge, all the survived mice were anatomized to check the pathological lesions of organs and tissues caused by the infection with virulent *E. coli*.

Statistical Analysis

Data from all groups were presented as $\bar{x}\pm s$ and compared by Student's test of variance analysis. $P<0.05$ was considered statistically significant.

RESULTS

Preparation and Detection of CNP

Transmission electron microscopy (Fig. 1) displayed that most of CNPs were spherical. The analyses of Zetasizer3000HS/IHPL showed that the average granule diameter was 45 nm, multi-dispersivity was 0.190 (Fig. 2), Zeta electronic potential was +25.6 mV, suggesting that the surface of CNP carried positive ions.

Gel Retardation Assay

Electrophoresis on 1.5% agarose gel showed that CNP-CpG in lane C did not move out of the sample hole and was entirely blocked. All CpGs were entrapped with chitosan (Fig. 3).

Stimulatory Effect of CpG ODN on Pig Lymphocytes in vitro

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

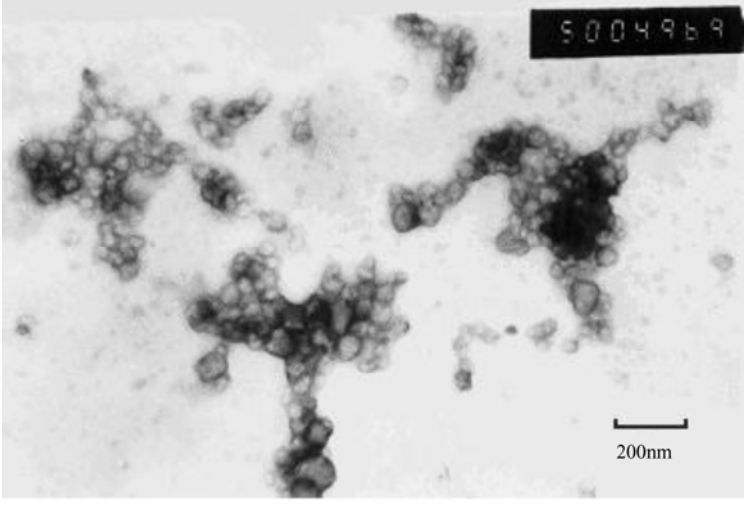


FIG. 1. Transmission electronic micrograph of chitosan nanoparticles (×50000).

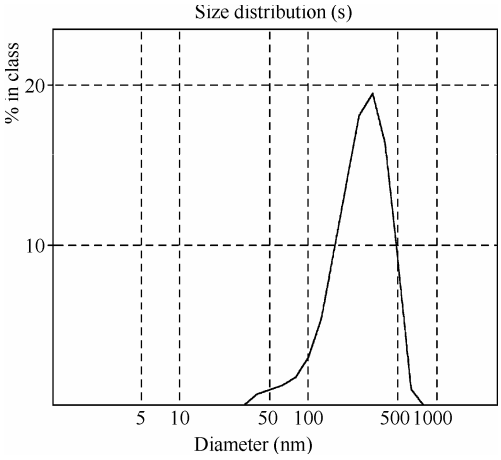


FIG. 2. CpG-chitosan nanoparticle size distribution.

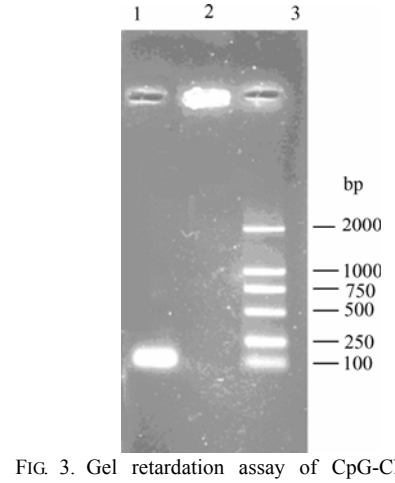


FIG. 3. Gel retardation assay of CpG-CNP (1.5% agarose electrophoresis). Lane 1: CpG; lane 2: CpG enwrapped with CNP; lane 3: DL2000 marker on 1.5% agarose gel electrophoresis.

TABLE 1

Stimulatory Effect of CpG on Immune Cells of Porcine *in vitro* ($\bar{x} \pm s$)

Treatment	OD_{570}
CpG	0.388 ± 0.059^{c}
E. coli	0.643 ± 0.107^{b}
CpG+*E. coli*	1.041 ± 0.189^{a}
Control	0.055 ± 0.015^{d}

Note. The data in the same column with different superscript letter are significantly different ($P<0.05$), and vice versa.

Amount of IgG, IgM, and IgA

The contents of total IgG, IgA, and IgM in the sera of immunized mice were significantly higher than those in the control group ($P<0.05$) after inoculation with the CpG sequence and CNP-CpG (Fig. 4). Among the four groups, inoculation with chitosan CpG nanoparticles resulted in the highest increase in IgG. But there were no remarkable differences between the oral and muscular administration groups with chitosan CpG nanoparticles ($P>0.05$). When the mice were challenged with virulent *E. coli* after 35 days, the contents of IgG, IgA, and IgM in the sera strikingly increased compared with those in controls ($P<0.05$).

Assay of Interleukin in Immunized Mice

The contents of IL-2, IL-4, and IL-6 in inoculated mice were significantly higher than those in controls ($P<0.05$) (Fig. 5). The differences between oral and intramuscular groups were not remarkable ($P>0.05$). After the mice were challenged with virulent bacteria, the contents of IL-2, IL-4, and IL-6 in inoculated groups were still notably higher then those in controls.

Changes of the Number of Immune Cells

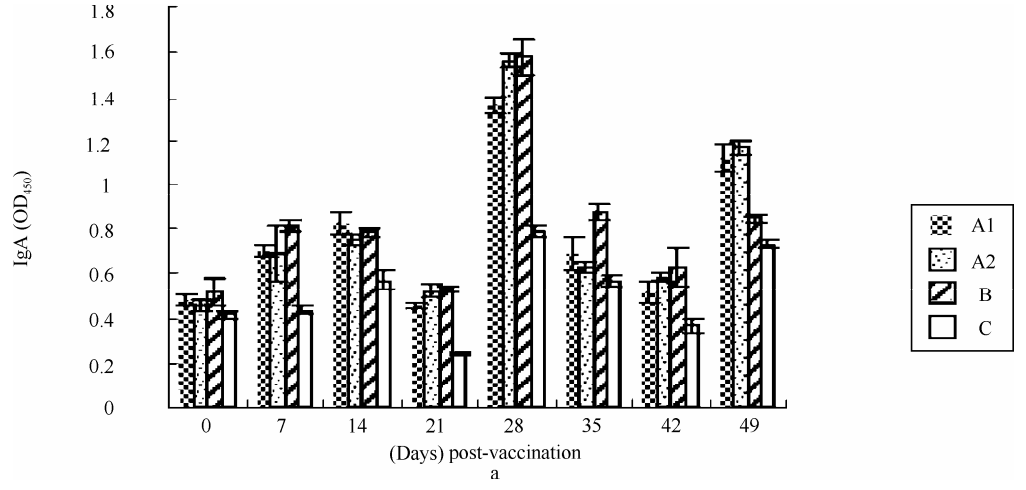
The number of immune cells significantly increased in the immunized mice compared to the control group ($P<0.05$) except for the number of neutrophils (Fig. 6). The number of immune cells was also higher in the mice inoculated with chitosan CpG nanoparticles than that in the naked CpG. group, but they were not significantly different ($P>0.05$).

Challenge Results

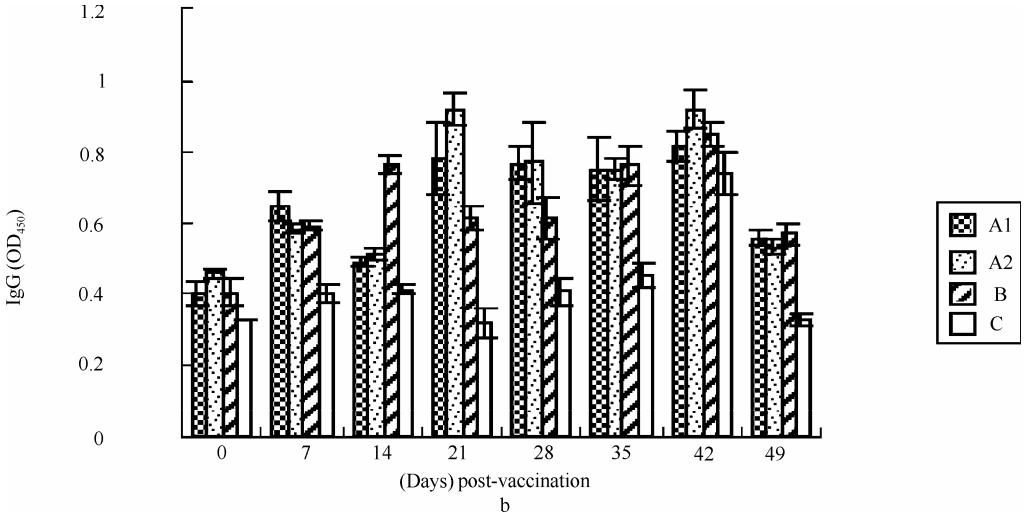
After orally fed with virulent *E. coli* for two weeks, the mice inoculated with CpG motifs or CNP-CpG survived, the control group was infected and manifested with low spirits with their hair sticking up, unusual and pliable brown stool. When all the survived mice were anatomized, no evident lesions were observed in the organs and tissues of immunized mice. The control mice all had severe lesions, including swelling, edema and necrosis of liver and spleen, catarrh and bleeding of stomach, duodenum and jejunum.

DISCUSSION

Adjuvant-like properties of CpG motifs have been observed in studies of antigen-encoding DNA plasmids, bacterial DNA or synthetic oligodeoxynucleotides (ODN)[19]. The immune stimulatory effects of ODN containing CpG dinucleotides depend entirely on the bases of CpGs, the number of CpGs and the space between the individual CpG motifs. Depending on different structures of CpG motifs and animal species, the adjuvant effect of CpG ODNs on vaccination varies greatly[20].



a



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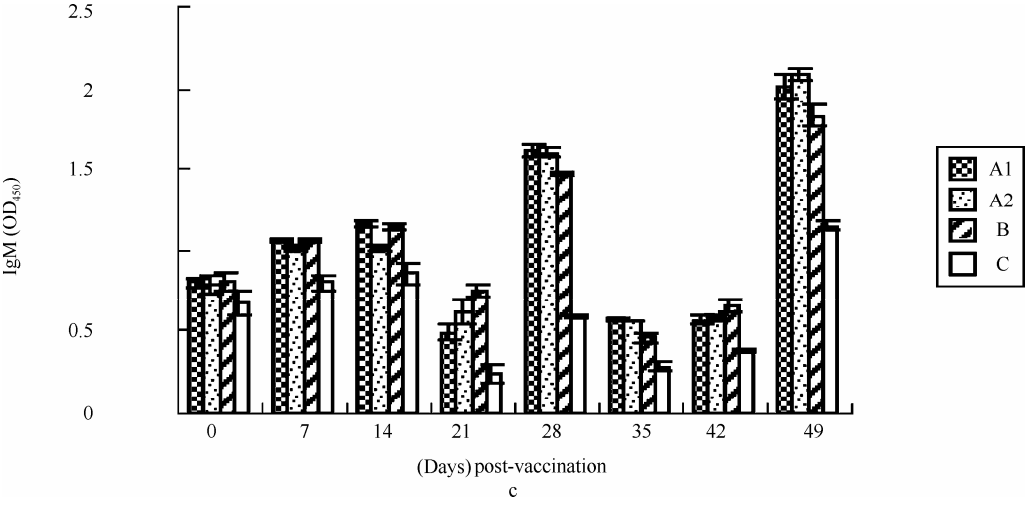


c

FIG. 4. Change of IgA, IgG, and IgM content in sera from experimental mice. A1 mice were intramuscularly injected with naked CpG DNA (30 pmol per mouse); A2 mice were intramuscular injected with CpG-CNP (6 pmol per mouse); B mice were orally fed with CpG-CNP (60 pmol per mouse); C mice were only injected with saline as in control group.

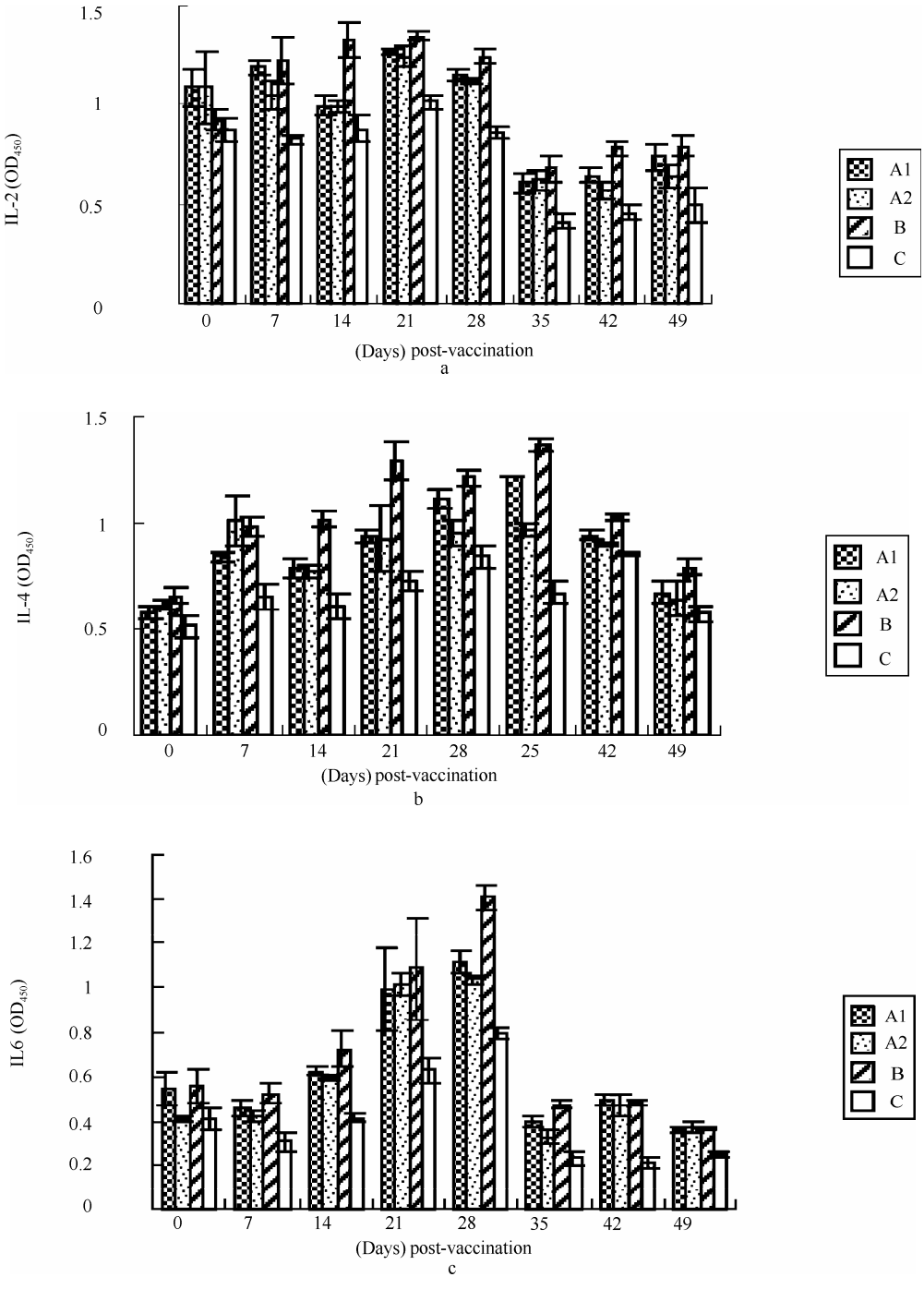
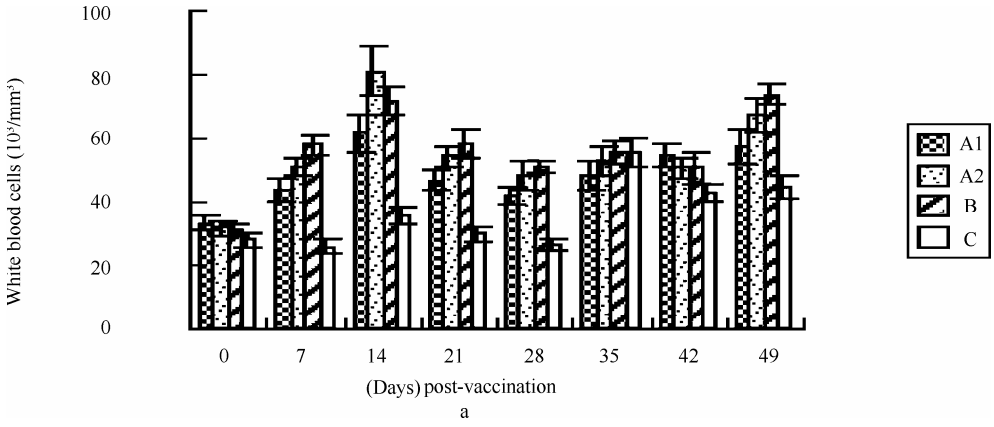


FIG. 5. Change of IL-2, IL-4, and IL-6 contents in sera from experimental mice.



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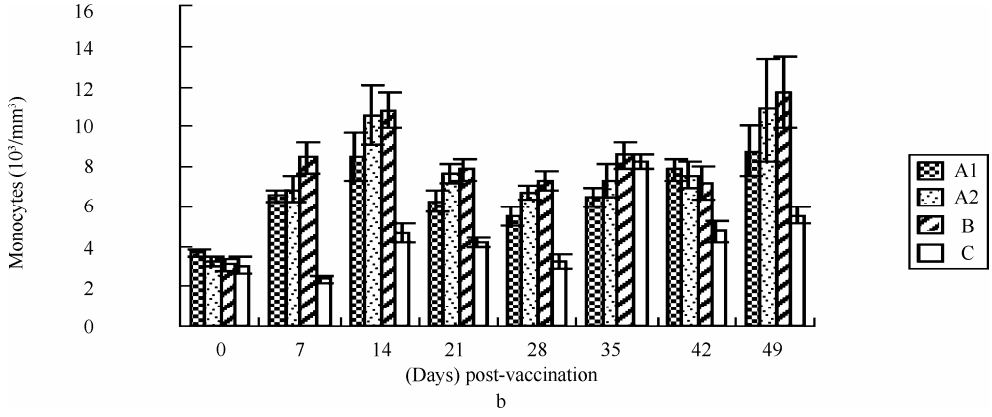

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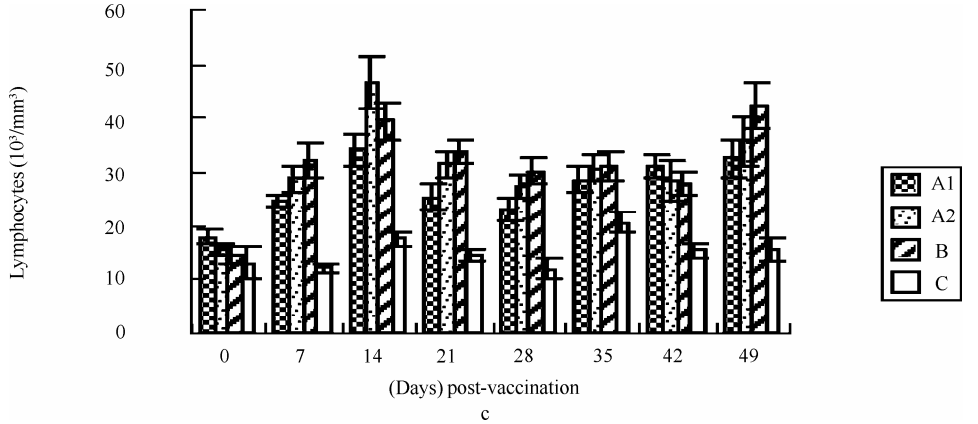

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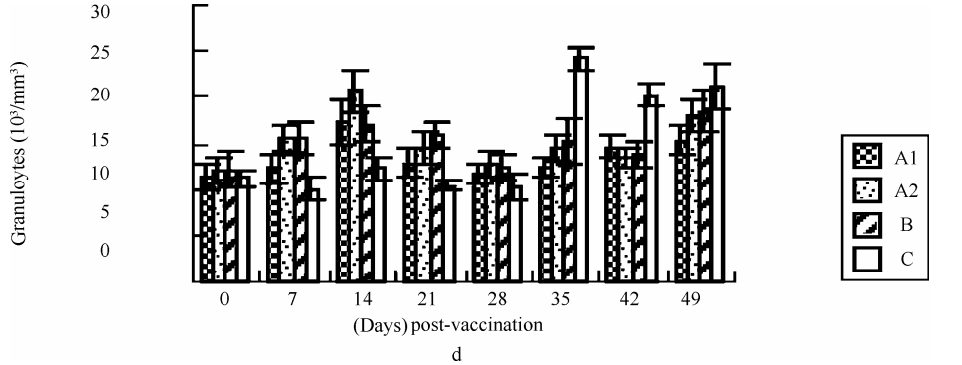

d

FIG. 6. Change of the number of white blood cells, monocytes, lymphocytes, and granuloytes in blood of experimental mice.

Our synthetic CpG sequence backbone contains 88 bases including eleven specific CpG motifs, which stimulate the cellular immunity of swine *in vitro*. The results indicate that these CpG motifs can be used as an effective immunoenhancer to increase the immunity of animals against infection.

One of the most important factors affecting the application of CpG motifs to regulate the immunity of animals is to effectively deliver the ODNs containing CpG motifs into the target cells to avoid the remarkable degradation of CpG motifs by a series of enzymes *in vivo*. Researches have been undertaken to find more effective transference methods because naked CpG motifs are easily degraded and only last for a relatively short time *in vivo*. A variety of approaches have been tried to carry CpG motifs, including liposomes and other high molecular particles[21-22].

Our results indicate that although the dosage of CpGs enwrapped with CNP is only one-fifth of that of the naked CpGs, there are no remarkable differences between the cellular and humoral immune responses in the two groups of mice. Surprisingly, the immunity and resistance of mice orally inoculated with CpG-CNP are comparable with those of mice muscularly vaccinated with CpGs, or CpGs-CNP. These findings suggest that the package of CpG motifs with chitosan nanoparticles effectively protects CpG ODNs against degradation by various enzymes *in vivo*, decreases the adsorption of CpGs by proteins, and the release rate of CpGs, and may help CpGs to penetrate through the barrier of cell membrane, which would result in more CpGs to combine the Toll-like receptors and trigger stronger immune responses *in vivo*. These findings have confirmed the protective effect of chitosan for ODNs as previously reported[23], indicating that oral administration of CpG *motifs* could be employed as an easy and economic method to elevate the immunity and resistance of animals, especially for prevention of infection via digestive and respiratory tract. The results prove that chitosan nanoparticles and CpG motifs can be used as an effective immune adjuvant to increase the immunity of animals against infection with a promising and extensive prospect in future.

Enteropathogenic Escherichia coli (*ETEC*) strains are a major cause of many diseases[24], and often cause diarrhea and death in neonatal and newly weaned animals and bring tremendous economical loss and public health problems[25]. Due to excess use of antibiotics, frequent mutations of *E. coli* and easy transfer of antibiotic resistance between different *E. coli* strains[26], an efficacious prophylactic and therapeutic vaccine of *E. coli* is needed. Our results indicate that the contents of immunoglobulins and cytokines, including IL2 and IL6 of Th1 type, and IL4 are significantly increased in the sera of mice vaccinated and challenged with virulent *E. coli*, so does the number of immune cells in the vaccinated mice, suggesting that co-administration of CpG and CNP can remarkably enhance the cellular and humoral immunity and resistance of mice against *E. coli* infection, the CNP -CpG motifs can control the infection of *E. coli* in animals by promoting their general immunity.

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