INDUCTION OF A PROTECTIVE IMMUNE RESPONSE AGAINST CHLAMYDOPHILA PSITTACI IN SPF CHICKEN FOLLOWING VACCINATION WITH OMP-1 DNA AND CPG OLIGONUCLEOTIDES AS AN ADJUVANT

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Received for publication April 30, 2007

Abstract

In the present study we determined whether CpG motifs could be used as an immune adjuvant for the Omp-1 DNA vaccine against Chlamydophila psittaci, based on the major outer membrane protein (MOMP), to induce the protection in specific pathogen free chickens against an intranasal challenge. Six sequences of phosphorothioate-CpG oligodeoxynucleotides (CpG ODNs) with strong immunostimulatory activity were designed to be examined. The CpG ODN with three GTCG TT repeats significantly promoted splenic lymphocyte proliferation and increased macrophage nitric oxide in a in vitro study. Birds vaccinated with CpG ODN and pcDNA3.1:MOMP induced moderate antibody response and higher lymphoproliferative stimulation while chickens primed-boosted with DNA alone did not. Furthermore, DNA vaccine plus CpG ODN accelerated chlamydial clearance in the spleen and lungs, and decreased inflammatory cellular infiltration. All above evidences show that CpG ODN can be used as an effective adjuvant with a DNA vaccine and this immunisation protocol resulted in an enhanced clearance of Chlamyphila psittaci.

Key words: chickens, Chlamyphila psittaci, CpG ODN, MOMP, immunisation.

Avian chlamydiosis is caused by the bacterium Chlamyphila psittaci (C. psittaci) and is highly prevalent in China poultry flocks resulting in considerable economic losses to agricultural industries throughout the world as well (3). Furthermore, it presents a significant zoonotic risk to humans, varying from unapparent to severe systemic disease with interstitial pneumonia and encephalitis (1). Up to now, no commercial vaccine is currently available for preventing C. psittaci infection. Previous immunisation experiments indicated the use of recombinant major outer membrane protein (MOMP) based DNA vaccine as a mean of preventing severe clinical signs and lesions in a turkey model of C. psittaci infection (15, 16). Higher antibody titres were not related to better protection and even had a negative effect on bacterial excretion (17). Oligodeoxynucleotides (ODN) containing CpG motifs mimic the ability of microbial DNA to activate the innate immune system and have been shown to stimulate antigen presenting cells and to predominantly induce secretion of Th1-type cytokines, such as IL-12 and IFN-γ (4, 5). In addition, results from clinical studies indicate that CpG administered with chlamydial MOMP has been shown to induce MOMP-specific Th1 type immune responses and to enhance protective immunity against Chlamyphila muridarum infection (2). In order to increase the potential response of C. psittaci vaccination, especially the cellular response, we evaluated different vaccination approaches using pcDNA3.1: MOMP, r-MOMP, and CpG motifs. Supposing that CpG will improve the immune response to DNA vaccine, results of our study will demonstrate the promise of using CpG as an alternative adjuvant against C. psittaci infection.

Material and Methods

Bacterial strain. C. psittaci CBJ2003 strain used in the study was originally isolated from broilers with severe pneumonia and was grown in yolk sacs of 7-day-old specific-pathogen-free (SPF) chicken embryos. The plaque forming units (pfu) of the C. psittaci strain was determined as 1×106 in McCoy cells.

In vitro study. Several CpG ODNs used in this study were selected basing on published reports (Table 1) and tested for their in vitro stimulatory effects and nitric oxide (NO) production. The isolation of chickens splenocytes and proliferative response was determined
as previously described (11). CpG ODN stimulating NO production was measured at concentration of 0.1, 1, 10 µmol/L by the Griess assay (6).

**DNA vaccine construction and r-MOMP.**

*Omp-1* gene (accession number: EF202608) was amplified from the extracted DNA, analysed, and cloned into the *EcoRI* site of the pcDNA3.1 vector (Invitrogen). The recombinant plasmid was transformed into *E. coli* DH5α competent cells, grown and purified as described previously (16, 19). DNA concentration was determined, confirmed, and stored at -20°C in 1mM Tris (pH 7.8) 0.1mM EDTA. The DNA was diluted in 0.9% saline prior to immunisation. The MOMP-encoding gene was cloned into expression vector pET-32a and expressed in *E. coli* BL21 with isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer (19). The fusion protein was purified and identified by Western blot analysis as described previously. The recombinant protein was diluted in 0.1mM Tris-HCl (pH 8.5) to a final concentration of 1 mg/mL.

**Immunisation procedure.** All the studies were performed on six-week-old SPF chickens (Beijing Vital Bridge Co.Ltd). Thirty chickens were randomly assigned to five groups and administered intramuscularly with different combinations of the pcDNA3.1: MOMP (hereinafter referred to as ‘DNA’), r-MOMP, and CpG ODN. Boosting injection occurred two weeks later. Chickens of group 1 were vaccinated with 100 µg of DNA and boosted with 200 µg of r-MOMP. Chickens of group 2 were immunised and boosted with 100 µg of DNA. Group 3 was co-immunised with 100 µg of DNA and 20 µg of CpG, 14 d later with the same immune regime. Group 4 chickens were primed and boosted with 100 µg of r-MOMP emulsified in Freud’s incomplete adjuvant. The control group (group 5) was placebo-vaccinated with pc-DNA3.1 vector alone. Two weeks after boosting, all the chickens were challenged at day 42 by an intraperitoneal injection of 1×10⁵ pfu of *C. psittaci*. Clinical signs, gross lesions, and presence of chlamydial antigens in tissue sections were examined. Animal care and experimental procedures were performed in compliance with the guidelines issued by the Beijing Laboratory Animal Administration Committee on Animal Care.

**Detection of antibody and lymphocyte proliferative responses.** Blood samples were collected before each immunisation (days 14 and 28) and just before the challenge infection (day 42) and one week later (day 49) for the detection of MOMP-specific antibodies by ELISA. ELISA plates were coated with 2 µg/well of r-MOMP in 0.05 M bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Serial dilution of serum were added to the wells followed by the addition to the wells of horseradish peroxidise (HRP) conjugated to rabbit anti-chicken IgG antibody (Southern Biotechnology Associates Inc) and incubation at room temperature for 1 h. After washing, 3, 3′, 5′-tetramethyl benzidine (TMB) substrate was added. The reaction was stopped after 10 min incubation by the addition of 2 M sulphuric acid and the optical density (OD) was read on a microplate reader at 450 nm. Positive antibody was defined as the highest dilution that gave a ratio greater than 2.1 between test serum and the negative control serum (10).

Peripheral blood leukocytes were isolated from blood samples by venepuncture from each group at day 14 after challenge. Lymphocyte proliferative tests were performed as previously described. The T cells were then stimulated for 48 h with 20 µl of concanavalin A (50 µg/mL, mitogen control), 20 µl of r-MOMP (50 µg/mL, specific antigen), 20 µl of pcDNA vector (50 µg/mL, negative control), and 20 µl of RPMI-10 (medium control), all in triplicate. Following the stimulation, the cell proliferation was assessed by addition of 20 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (5mg/mL, MTT, Sigma) solution, prepared in PBS at a final concentration of 5 mg/mL to each well. Following incubation for 2 h, 100 µl of dimethyl sulphoxide (78.1 mg/mL, DMSO, Sigma) was added to each well, incubated overnight at 37°C and then OD read at 570 nm. Results were expressed as the stimulation index (SI), calculated as mean of the readings for antigen stimulated wells divided by mean for medium control wells.

**Table 1**

<table>
<thead>
<tr>
<th>ODN code</th>
<th>Sequence</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN1</td>
<td>5′-TCGTCGTTTTTGTGCGTTTGTGCGTTT-3′</td>
<td>Cattle, chickens, pigs</td>
<td>(4, 5, 7, 14)</td>
</tr>
<tr>
<td>ODN 2</td>
<td>5′-TCCATGACGTCTCTGTACGTGT-3′</td>
<td>Mice, rabbits</td>
<td>(2, 13)</td>
</tr>
<tr>
<td>ODN 3</td>
<td>5′-GTCGTT GTGCGTT GTGCGTT-3′</td>
<td>chickens</td>
<td>(18)</td>
</tr>
<tr>
<td>ODN4</td>
<td>5′-GTCGTT GTGCGTT GTGCGTTGAGGGGGG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ODN5</td>
<td>5′-TCCATGTCGTTTCTGTAGCGTTT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5′-TCGTCGTTTTTGTGCGTTTGTGCGTTT-3′</td>
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</table>

Analysed CpG-containing oligonucleotides (CpG ON)
Histological examination of the lungs and spleen. The remaining chickens were euthanised 14 d after challenge infection. The spleen and lungs were examined for gross lesions, and then fixed in 10% neutral formalin, embedded into paraffin blocks, and sectioned at 5 µm. Streptavidin-biotin peroxidase (SPTM kit, ZYMED, USA) procedure was used for immunohistochemical staining of chlamydial inclusion bodies. All steps of manipulation were carried out according to the manufacturer's instructions of the kits. The sections were assessed semi-quantitatively by one experienced observer.

All data were expressed as mean ± standard error. The Student’s *t*-test was employed for all statistical analyses. Results were considered significantly different at the level of *P*<0.05.

Results

Lymphocyte proliferation and NO production in an *in vitro* study. The strong splenocyte proliferation occurred after stimulating with CpG ODN1, CpG ODN3, CpG ODN4 and CpG ODN5, respectively. CpG ODN1 stimulated significantly the lymphocyte proliferation. In contrast, CpG ODN 2 induced a lower stimulation index as compared to CpG ODN3 and CpG ODN4 (Fig.1). In addition, CpG ODN 1 induced the highest lymphocytes proliferative response, which was correlated to the results of NO production. In the study, NO production was significantly higher in the groups with 3 CpG motifs of 5’GTCGTT3’ than that in the CpG ODN5 with 2 CpG motifs. Moreover, lymphocyte proliferation and NO production revealed dose-dependent manners in all tested groups (Fig. 2). Based on the results of the *in vitro* assays, CpG ODN1 was selected as adjuvant candidate for *in vivo* application.

Humoral responses induced by immunisation. The MOMP antibody titres for all groups of chickens are shown in Fig. 3. Two weeks after inoculation, there was obvious increase in the titres of specific antibodies. At days 14, 28 and at day after challenge, the group immunised with DNA and CpG ODN1 had significantly higher titres than the DNA vaccine group. At day 14 after challenge, combination of DNA with CpG ODN induced also higher titres than the group primed with DNA followed by r-MOMP did. The level of antibody induced by DNA or vector alone was significantly lower than other approaches.

T cell response induced by immunisation. ConA mediated lymphocyte proliferation was measured by the MTT assay. Representative results of a proliferation study using lymphocytes of various groups were showed in Fig. 4. At day 42, the chickens immunised with DNA and CpG ODN resulted in a significantly higher stimulation index than that of the other groups, suggesting a possible effect of the CpG in vaccination. Chickens priming with DNA followed by MOMP and priming boosting with DNA alone also induced some higher stimulation index than the group that received r-MOMP alone. In comparison to the T cells response of groups 1 and 2, chickens in group 2 had an obviously higher stimulation index.

Clearance of *C. psittaci* in the lungs and spleen. The induced protection was evaluated by semi-quantification of *C. psittaci* in the lungs and spleen. Chlamydia quantity in chickens co-immunised with DNA and CpG was significantly lower than in the other observed groups, except for chickens inoculated with DNA alone. The above result was confirmed by small inclusion numbers in immunohistochemical staining. The sections of lungs revealed inclusion bodies in all immunised birds (Table 2). The best protection was observed in the group co-immunised with DNA and CpG, which displayed the lowest number of inclusion bodies. However, when overall staining scores between groups 2 and 3 were compared, there was less bacterial replication in group 3 than in group 2. There were higher numbers of inclusions in group 4 compared to group 2. The clearance effect was correlated to higher proliferative responses of spleen lymphocytes in group 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Chlamydia inclusion in immunohistochemical staining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slightly congested</td>
<td>Slightly enlarged</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>Focal consolidation</td>
<td>Moderately enlarged</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>Focal infiltration</td>
<td>Slightly enlarged</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Extensively congested</td>
<td>Moderately enlarged</td>
<td>+++</td>
</tr>
<tr>
<td>Control</td>
<td>Exudative pneumonia</td>
<td>Severely enlarged</td>
<td>++++</td>
</tr>
</tbody>
</table>

*Chlamydial inclusion in the spleen and lungs under microscope.
Fig. 1. Chicken splenocytes proliferation in response to 0.1, 1.0 and 10.0 µmol/mL CpG ONDs \textit{in vitro}.

Fig. 2. Nitric oxide production in spleenocytes stimulated with 0.1, 1.0 and 10.0 µmol/mL CpG ONDs \textit{in vitro} study.

Fig. 3. Systemic MOMP antibody response after immunisation.
Animal sera were taken on days 14, 28, 42 (challenge), and 49 (7 d after challenge).
Discussion

The adjuvant effect of CpG ODN has been well recorded by many laboratories in mice and humans. The ability of CpG ODN to induce both innate and adaptive cellular immune response has made it a potential treatment or prophylactic vaccine adjuvant for disease relying on cellular immunity (7, 8, 13, 14). In the current study, CpG ODN with strong immunostimulatory activity in vitro was firstly carried out using 6 sequences of phosphorothioate-CpG ODNs according to the previous reports in different species. CpG ODN1 with three GTCGTT repeats stimulated lymphocyte proliferation and increased macrophage NO production in a higher degree than CpG ODN 3 and CpG ODN 4 did. It has been shown that the GTCGTT motif is optimal for the stimulation of lymphocyte proliferation in several species, including cattle, sheep, goats, horses, pigs, and chickens (7, 8, 14, 20), while the GACGTT motif was optimal only for inbred rabbits and mice (2, 13). In this study, the sequence with three GTCGTT repeats connected by TT is able to induce a stronger lymphocyte proliferation and enhance macrophage NO production while the sequence with similar repeats without TT cannot induce a higher NO production. Our findings were in disagreement with the previous reports indicating that both CpG ODN1 and CpG ODN 3 were able to induce higher NO production. The difference is due to the primary macrophage cell in this study, instead of the HD11 macrophage cell lines as previous described (18).

Previous studies showed that DNA immunisation appears to be a promising method in preventing C. psittaci infections since DNA vaccines have already been used with more or less success in C. psittaci vaccination models (15, 16). In order to develop more efficient vaccination regimens, protection was evaluated when vaccination with plasmid DNA was followed by that with r-MOMP (17). In addition to antibodies, cell-mediated immunity plays an important role in immunity against C. psittaci infection (12). Co-immunisation with CpG ODN1 and DNA vaccine is able to enhance specific antibody response and induce a strongly cellular immune response against virulent C. psittaci in SPF chickens, as shown by the reduction of Chlamydia quantity in the lungs and spleen. In the present study, immunisation of chickens with DNA followed with r-MOMP also stimulated higher antibody levels as well as moderate lymphocyte proliferation indexes, and an insufficient protection was observed by the reduction of the mean Chlamydia quantity in the lungs. This outcome is in accordance with the previous reports that chickens were partially protected (9, 17). Our results indicated that DNA vaccination with CpG ODN1 might have potential as an adjuvant for chlamydial DNA vaccine.

Moreover, our study is different from a recent report indicating that immunisation with DNA and CpG created no beneficial effect on the protective immune response in turkeys (9). The main cause is due to a mix of two sequences as adjuvant candidate and a lower dosage used in the previous study. Although CpG ODN 2006 and CpG ODN ML02 are able to stimulate NO individually in the previous study, a mix for in vitro study is not elucidated and its function is unclear.

Avian chlamydiosis has been a serious disease in poultry industry; the pathogen is able to persist in chickens and evades the immune system. The combination with DNA and CpG is able to elicit an active immune response of DNA vaccine based on MOMP. Further studies would be required to evaluate the secretion of various cytokines and interferon and acquire the mechanisms of the immunity induced by the DNA vaccine and action of CpG ODN in chickens.

Acknowledgments: This work was supported in part by the National Natural Science Foundation of
China (No. 30370070) and the Beijing Municipal Science and Technology Commission (No. 6052014) to Dr. Cheng He.

References