In ovo administration of CpG oligodeoxynucleotides and the recombinant microneme protein MIC2 protects against *Eimeria* infections

Rami A. Dalloul\(^a\), Hyun S. Lillehoj\(^{a,*}\), Dennis M. Klinman\(^b\), Xicheng Ding\(^a\), Wongi Min\(^c\), Robert A. Heckert\(^a\), Erik P. Lillehoj\(^d\)

\(^a\) Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, BARC-East, Building 1040, ARS, USDA, Beltsville, MD 20705, USA
\(^b\) Section of Retroviral Immunology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA
\(^c\) Department of Animal Science, Sunchon National University, 315 Maegok-Dong, Suncheon, Chonnam 540-742, Korea
\(^d\) Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, MD 21201, USA

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Abstract

We have previously demonstrated that short oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs) exert a positive effect on weight loss and oocyst shedding associated with *Eimeria* infection when injected in vivo. The present work investigated the effects of in ovo vaccination with CpG ODNs and an *Eimeria* recombinant microneme protein (MIC2), alone or in combination, on susceptibility to coccidiosis. In ovo injection of CpG ODNs alone enhanced resistance to experimental *Eimeria acervulina* infection as best exemplified by reduced oocyst shedding. Two CpG ODNs reduced the oocyst load, but did not affect weight gain. When co-administered with the recombinant microneme protein, both ODNs reduced oocyst shedding; however, only ODN D19 plus MIC2 consistently improved weight gain. Vaccinating with ODN 2006 or MIC2 protein curtailed oocyst shedding but did not enhance weight gain in *Eimeria tenella*-infected birds. Co-administration of CpG ODN and MIC2 did not have an additive effect in reducing the oocyst output; however, it resulted in the highest and lowest Ab response before and after *Eimeria tenella* infection, respectively. Collectively, CpG ODNs administered in ovo demonstrated immunoenhancing and adjuvant effects following *Eimeria* infections.

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Keywords: CpG; Oligodeoxynucleotide; In ovo vaccination; *Eimeria*

I. Introduction

Livestock vaccination remains the most effective method for preventing infectious diseases and reducing associated economic losses. This is particularly true for avian coccidiosis, a major parasitic disease of substantial economic cost for the poultry industry that has relied upon prophylactic medication in the absence of efficient vaccines [1,2]. Alternative disease control strategies including novel and effective vaccines are needed to control coccidiosis especially with the emergence of antigenic variants of *Eimeria*, the etiologic agent [3]. The use of avian vaccines administered in ovo has been reported both experimentally and commercially for several diseases. The advantages of in ovo vaccination include early immunity, reduction in bird stress, precise and uniform dosage, multiple-agent vaccination, and ease of handling and reduced labor costs. Since it was first reported by Sharma and Burmester [4] for Marek's disease, in ovo vaccination has been routinely and efficiently administered for some pathogens but less successful for others including those for coccidiosis.

Short oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG ODNs) have been shown to
be effective immunoprotective agents and vaccine adjuvants in mammalian systems, by inducing both innate and adaptive immune responses [5,6]. Prior administration of CpG ODNs was found to protect mice against challenge with the intracellular pathogens Leishmania major [7,8], Listeria monocytogenes [9], and Francisella tularensis [10]. The adjuvant effect of CpG ODNs to a subunit vaccine against malaria parasite has been observed [11]. We recently reported that in vivo injection of CpG alone protected against certain Eimeria infections [12]. Others demonstrated the immunostimulatory effects of CpG ODNs on humoral immune responses [13] and protection against Escherichia coli [14]. Furthermore, CpG ODNs were shown to enhance the immunogenicity of conventional protein antigens [15], such as ovalbumin, as manifested by a threefold increase in antibody production when co-injected with the protein [16]. Consequently, we hypothesized that in ovo injection of CpG ODN with or without a recombinant Eimeria protein would be as effective as in vivo vaccination, especially since the former method is more practical for commercial application in the poultry industry. The goals of these trials were to evaluate the in ovo protective effects of CpG ODNs alone and as vaccine adjuvants for Eimeria in specific pathogen free (SPF) chickens.

2. Materials and methods

2.1. Experimental birds

All live bird studies were conducted in compliance with the USDA animal use procedures and protocols. In all experiments, SPF fertilized eggs of White Leghorn chickens were purchased (SPAFAS, Charles River, CT) and hatched at the Animal and Natural Resources Institute (USDA, Beltsville, MD). Hatched birds were wing-tagged, and feed and water were provided ad libitum throughout the experimental period. SPF fertilized eggs of White Leghorn chickens were incubated with continuous gentle shaking, washed with PBS-1% BSA, and blocked with PBS-1% BSA. Serum dilutions (1:2–1:64; 100 μl/well) were added, incubated with continuous gentle shaking, washed with PBS-

2.2. Oligodeoxynucleotides, protein, and in ovo injection

Oligodeoxynucleotides were designed using published sequences as shown in Table 1, and were synthesized with a phosphorothioate backbone. Each CpG ODN was appropriately diluted in sterile phosphate-buffered saline (PBS), mixed with the recombinant Eimeria microneme protein MIC2 [17] where needed, and administered in ovo into the amniotic cavity of 18-day-old embryos in a 100 μl volume using a customized Intelliject in ovo injection system (AviTech LLC, Hebron, MD). MIC2 is derived from a protein found in coccidia micronemes and involved in host cell attachment and penetration [18]. Injected doses of CpG ODNs and MIC2 protein varied among experiments as indicated in each figure legend.

2.3. In vitro nitric oxide assay

All CpG ODNs were screened for in vitro activity using a chicken macrophage cell line (HD11) prior to live bird studies. Cells were cultured in 24-well plates at a concentration of 5 × 10⁵/well (1 ml) and one milliliter of appropriate stimulant added. Nitric oxide production by HD11 cells was assessed in triplicate wells of 96-well plates as nitrite content in conditioned media using Griess reagent as described [19,20]. Sodium nitrite was used as the standard and ConA as the stimulant positive control.

2.4. Eimeria infection and assessment of fecal oocyst production

Prior to infection, all experimental birds were reared in brooder pens in Eimeria-free facility and transferred into small cages in separate location where they were infected and kept until the end of experimental period. At six days of age, each bird (except for those of the negative control groups) received an oral dose of 10,000 sporulated oocysts of either Eimeria acervulina (EA) or Eimeria tenella (ET). Fecal materials were collected 5/6–9 days post infection (dpi), processed, and the number of shed oocysts counted. Oocyst production and shedding were assessed as described by Dalloul et al. [21] where the number of oocysts per bird was calculated using the formula: [total number oocysts = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/number of birds per cage]. All birds were individually weighed at 0, 5/6, and 9 dpi. Oocyst production and body weight gains were selected as best indicators of susceptibility to coccidiosis [22].

2.5. ELISA for serum antibody levels

Serum samples collected prior to infection (9 days post in ovo injection) and 9 dpi were tested for anti-coccidial Abs using ELISA as described [21]. Briefly, microtiter plates were coated overnight with 1 μg/well of the recombinant coccidial antigen MIC2, washed, and blocked with PBS-1% BSA. Serum dilutions (1:2–1:64; 100 μl/well) were added, incubated with continuous gentle shaking, washed with PBS-

Table 1

<table>
<thead>
<tr>
<th>ODN code</th>
<th>Sequence (5′-3′)</th>
<th>Length (bases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-CpG control</td>
<td>GCTGTGCTTTGGTCTTTTGGTCTT</td>
<td>24</td>
</tr>
<tr>
<td>2006</td>
<td>GTGCTGTGCTTTGGTCTTTTGGTCTT</td>
<td>24</td>
</tr>
<tr>
<td>Ma (1:1)</td>
<td>1466</td>
<td>TCAACGTGGTA</td>
</tr>
<tr>
<td>1555</td>
<td>GCTAGCGCAGCAG</td>
<td>15</td>
</tr>
<tr>
<td>K Ma (1:1)</td>
<td>K3</td>
<td>ATGCAGCCTGAGGGTTCTC</td>
</tr>
<tr>
<td>K123</td>
<td>TCGTTCGTTCTC</td>
<td>12</td>
</tr>
<tr>
<td>D19</td>
<td>GGTGCA TCGA TGCAGGGGG</td>
<td>20</td>
</tr>
</tbody>
</table>
0.05% Tween, and bound Ab detected with peroxidase-conjugated rabbit anti-chicken IgG (Sigma) and peroxidase-specific substrate at 450 nm.

2.6. Statistical analysis

Differences between experimental treatments were tested by one-way ANOVA (GraphPad InStat, GraphPad Software Inc., San Diego, CA) and considered significant at *P* < 0.05, and mean values were then compared by the Tukey–Kramer multiple comparisons test. An asterisk in the figures indicates significant differences compared with the positive control (PBS-injected and EA- or ET- infected groups).

3. Results

3.1. CpG ODNs induced nitric oxide secretion in HD11 cells

Screened by nitric oxide production, several CpG ODNs induced nitric oxide secretion by activated HD11 cells (Fig. 1). Their stimulatory effects were dependent on the dose and sequence motif, and only a single dose for each ODN is shown at 24 h following HD11 stimulation (Fig. 1). The four presented CpG ODNs (2006, murine Mix [1466 + 1555], K Mix [K3 + K123], D19) were used for subsequent in ovo experiments.

3.2. Effects of CpG ODNs on oocyst shedding and weight gain in EA-infected birds

The four CpG ODNs and a non-CpG ODN were injected at 25 or 50 μg/embryo. Among all treatments, only ODNs Mix and D19 resulted in reduced oocyst shedding at 25 and 50 μg, respectively (Fig. 2). There was no significant effect on weight gain of any CpG treatment when compared to the infected control group.

3.3. CpG ODNs with MIC2 protein reduced oocyst output and improved weight gain

Embryonated eggs were injected with 10, 33, or 100 μg MIC2 protein in combination with CpG ODN, Mix or D19, at 25 μg each and body weight gain and oocyst shedding determined as parameters of infection following EA challenge. ODN D19 significantly improved weight gain (Fig. 3 B) and reduced oocyst shedding (Fig. 3 A) with all three protein doses, with the MIC2 dose of 33 μg/bird showing the best effect in reducing oocyst load. Similarly, ODN Mix in conjunction with either 33 or 100 μg MIC2 protein reduced the oocyst load (Fig. 3 A), and only the 10 μg MIC2 + Mix improved weight gain (Fig. 3 B).

3.4. Effect of ODN 2006 and MIC2 injection on oocyst shedding and weight gain in ET-infected birds

On the basis of preliminary findings and since MIC2 was originally derived from *E. tenella*, we also injected MIC2...
alone or with ODN 2006 and later infected with ET. The recombinant protein MIC2 and CpG ODN 2006, alone or combined, reduced oocyst shedding in ET infected birds (Fig. 4A). However, there was no significant effect for any of the treatments on weight gain (Fig. 4B).

3.5. Serum Ab response in birds injected with ODN 2006 and MIC2 before and after ET infection

As shown in Fig. 5A, slightly higher serum Eimeria-specific Abs were detected at 9 days post-in ovo injection and prior to ET infection in chickens injected with MIC2 and ODN 2006 alone. Yet, significantly higher Ab levels were observed in birds injected with both immunogens (Fig. 5A). Following ET infection, only the non-injected and MIC2-injected groups exhibited an increased humoral response compared to the non-infected controls (Fig. 5B). Further, the MIC2 + 2006 group had the lowest Ab levels.

Fig. 3. Effect of CpG ODNs and MIC2 protein injection on oocyst output (A) and body weight gain (B) 6–9 days following E. acervulina infection. Embryos (18-days-old, n = 10/group) were injected into the amniotic cavity with MIC2 protein (10, 33, or 100 μg/H9262 g), and hatched chicks (n = 9/group) were orally inoculated with 10,000 E. acervulina oocysts at 6 days of age. *P < 0.05; error bars = S.E.

Fig. 4. Oocyst shedding (A) and body weight gain (B) by chicks 5–9 days following E. tenella infection. Embryos (18-days-old, n = 10/group) were injected into the amniotic cavity with MIC2 protein (100 μg/H9262 g), ODN 2006 (25 μg), or MIC2 (100 μg) plus ODN 2006 (25 μg), and hatched chicks (n = 9/group) were inoculated with 10,000 E. tenella oocysts at 6 days of age. *P < 0.05; error bars = S.E.

Fig. 4. Oocyst shedding (A) and body weight gain (B) by chicks 5–9 days following E. tenella infection. Embryos (18-days-old, n = 10/group) were injected into the amniotic cavity with MIC2 protein (100 μg/H9262 g), ODN 2006 (25 μg), or MIC2 (100 μg) plus ODN 2006 (25 μg), and hatched chicks (n = 9/group) were inoculated with 10,000 E. tenella oocysts at 6 days of age. *P < 0.05; error bars = S.E.

4. Discussion

The present work investigated the effects of in ovo vaccination with CpG ODNs and an Eimeria recombinant microneme protein, alone or in combination, on susceptibility of chickens to coccidiosis. In ovo injection of CpG ODNs alone enhanced resistance to experimental E. acervulina infection as best exemplified by reduced oocyst shedding. Two CpG ODNs at different concentrations, Mix and D19, reduced the oocyst load, but did not affect weight gain. When co-administered with the recombinant microneme protein, both ODNs reduced oocyst shedding, with the ODN Mix plus MIC2 showing a direct relationship between weight gain and oocyst output. However, only ODN D19 plus MIC2 improved weight gain, proving to be the more effective combination in protecting against E. acervulina infection. Vaccinating with ODN 2006 or MIC2 protein curtailed oocyst shedding but did not enhance weight gain in E. tenella-infected birds. Co-administration of CpG ODN and MIC2 did not have an additive effect in reducing the oocyst output; however, it resulted in the highest and
Fig. 5. Ab response of SPAFAS chickens before and 9 days following *E*. *tenella* infection. Embryos (E18, \( n = 10 \)/group) were injected into the amniotic cavity with MIC2 protein (100 \( \mu \)g), ODN 2006 (25 \( \mu \)g), or MIC2 plus ODN 2006, and hatched chicks (\( n = 9 \)/group) inoculated with 10,000 *E*. *tenella* oocysts at 6 days of age. (Reported serum dilution is 1:4) *P* < 0.05; error bars = S.E.

Ideally, the most effective method to manage infectious diseases is through vaccination with the most appropriate immunogen/adjuvant combination to stimulate innate and adaptive immunities. The innate immune system recognizes conserved microbial components (pathogen-associated molecular patterns, or PAMPs) present in a wide variety of infectious agents using cellular components including Toll-like receptors (TLRs) [23]. Stimulation of TLRs by microbial products not only is sufficient for activation of adaptive immune responses, but also is often required for induction of Th1 effector responses [24]. It is therefore possible to stimulate protective immunity by a single potent PAMP (e.g., CpG ODN) or, more commonly, using the PAMP as an adjuvant in combination with immunogens.

The most prominent symptom of avian coccidiosis is growth retardation characterized by reduced weight gains, or weight loss in severe cases, with major economic cost to the poultry industry. In earlier work, we reported that in vivo administration of an eimerian recombinant protein or its encoding cDNA induced protective immunity against EA infection [25]. Subsequently, we showed that protection against *Eimeria* infection developed following in vivo administration of ODN 2006 [12]. In the current study, we showed for the first time that this and other CpG ODNs, whether injected alone or with an immunogen, provided defense against two *Eimeria* infections when administered in ovo.

Lack of a commercially effective coccidiosis vaccine, coupled with the emergence of drug resistant strains of *Eimeria*, has prompted poultry scientists to investigate alternative approaches to the current coccidiosis control protocols [1,3,26,27]. One such avenue is in ovo vaccination. In ovo vaccination has been successfully used in the poultry industry for many diseases [28–30], mostly viral, and use of this technology for coccidiosis has been recently reported. In ovo vaccination with *Eimeria* oocysts was reported to stimulate immunity to a subsequent live challenge in broiler chickens [31,32]. However, these initial studies examined vaccination with live or attenuated *Eimeria* oocysts with limited protection spectrum and, not unexpectedly, only partial immunity was achieved. Therefore, we were interested in examining whether in ovo vaccination with CpG ODNs plus a recombinant protein would induce more complete protective immunity. As discussed above, we observed that in ovo vaccination with one CpG in particular (D19) plus MIC2 improved weight gain and reduced oocyst shedding upon *Eimeria* challenge infection. In mammals, CpG ODNs of the D-type enhance the function of antigen presenting cells and have shown protective effects against intracellular parasitic infection [8], and are also known to support Th1-like immune responses when used as adjuvant in primates [33].

The immunomodulatory effects of CpG ODNs are known to vary, according to the route of delivery and host species [34]. As an additional variable described in this study, anti-coccidial Ab responses in ET-infected chickens were highest with co-injection of CpG ODN and the recombinant protein before *Eimeria* infection, but were lowest after infection despite reduction in oocyst shedding. Similar to mammals [33], Th1-like immune responses in vivo may explain the improved resistance of chickens injected with CpG ODN (alone or with protein) in the presence of a low humoral response. However, the occurrence of protective Abs in coccidiosis remains debated, and a more prominent role for cellular immunity in protection against reinfection has been advocated [1,27]. However, the mechanistic details mediating such protection are not fully understood and remain to be clarified, especially in light of the wide array of immune cells activated by CpG ODNs and the diversity of immune responses induced.

In conclusion, we showed that CpG ODNs administered in ovo demonstrated immunoenhancing, adjuvant effects upon *Eimeria* infections. ODN D19, whether alone or mixed with the recombinant protein, reduced oocyst shedding and improved weight gain, suggesting that it may be the best ODN candidate for future studies in our coccidiosis vaccine development program. Current investigations are focused on optimizing vaccination parameters such as adjuvant dosage...
and delivery schedule, as well as testing against other *Eimeria* species.

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References


