Montanide™ ISA 71 VG adjuvant enhances antibody and cell-mediated immune responses to profilin subunit antigen vaccination and promotes protection against *Eimeria acervulina* and *Eimeria tenella* ©

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**Abstract**

The present study was conducted to investigate the immunoenhancing effects of Montanide™ ISA 71 VG adjuvant on profilin subunit antigen vaccination. Broiler chickens were immunized subcutaneously with a purified *Eimeria acervulina* recombinant profilin protein, either alone or mixed with ISA 71 VG, and host immune responses were evaluated. After secondary immunization, antigen-specific antibody and T-cell responses were higher in the group which received profilin plus ISA 71 VG compared with the other groups. Furthermore, body weight gains and fecal oocyst shedding were evaluated following oral challenge infection with live *E. acervulina* or *Eimeria tenella* oocysts. Vaccination with profilin plus ISA 71 VG reduced oocyst shedding compared with animals immunized with profilin alone. These results demonstrate that the recombinant profilin subunit vaccine, when given in combination with Montanide™ ISA 71 VG, augments protective immunity against *E. acervulina* and *E. tenella*.

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1. Introduction

Avian coccidiosis is an economically important parasitic disease caused by intestinal infection by intracellular protozoa belonging to several species of the genus *Eimeria* (Lillehoj and Lillehoj, 2000; Lillehoj et al., 2000). Chickens infected with coccidia parasites exhibit multiple clinical symptoms, among the most important being decreased feed utilization, reduced body weight gain, and fecal shedding of infectious oocysts that re-infect susceptible animals upon ingestion. Avian coccidia are highly immunogenic, and primary infections induce a robust protective immune response to challenge infection by the homologous parasite (Allen and Fetterer, 2002). Although live parasite vaccines and drugs are primarily used for disease control in commercial production facilities, there is an increasing need to develop alternative strategies due to the emergence of drug resistant coccidial strains and complications with clostridial infections associated with live coccidia vaccines (Chapman, 1984; Lillehoj et al., 2000; Park et al., 2008).

One of the most effective methods to manage infectious diseases in veterinary medicine is through the induction of protective innate and adaptive immunities by antigen vaccination in combination with adjuvant (Cox and Coulter, 1997). Veterinary adjuvants, such as those based on water-in-oil (W/O) emulsions, have been successfully applied to enhance antigen-specific immune responses for more than 80 years (Bowersock and Martin, 1999; Newman and Powell, 1995). Among the candidate oil-based adjuvants, the Montanide ISA series of emulsions have shown good efficacy for generating protective immunity against major veterinary diseases when used in combination with recombinant proteins derived from the infectious agent (Aucouturier and Ganne, 2000). In spite of these encouraging results, however, there is a paucity of adjuvants that have been approved for use in poultry.

Given the previous success with Montanide adjuvants in bovine, porcine, and ovine settings (East et al., 1992; Iyer et al., 2001), we conducted a series of studies to evaluate the immunoenhancing effects of these W/O emulsions in the context of experimental avian coccidiosis. Montanide™ ISA 71 VG (ISA 71 VG) is a mineral oil-based adjuvant that has been developed for the manufacture of water-in-oil (W/O) emulsions. It is consisted of an enriched light mineral oil with an extremely refined emulsifier obtained from...
mannitol and purified oleic acid of vegetable origin. ISA 71 VG was specifically formulated to stimulate cell-mediated immunity. In contrast, ISA 70 VG which is consisted of a high grade injectable mineral oil and is used in the manufacture of W/O emulsions, was not specifically designed for triggering a cellular response.

The 3-1E gene encodes *Eimeria* profilin which is highly conserved across different stages of *Eimeria* life cycle and multiple *Eimeria* species such as *Eimeria tenella* and *Eimeria maxima* (Song et al., 2000). Our previous results demonstrated that immunization of chickens with a recombinant profilin protein derived from *Eim*-eria sporozoites in combination with ISA 70 VG increased body weight gains in *Eimeria acervulina*-infected animals compared with vaccination with profilin alone (Jang et al., 2010). In that study, however, only partial protection against challenge infection was obtained, since fecal oocyst shedding was unaffected by vaccination with profilin plus ISA 70 VG compared with profilin alone. Vaccination of chickens with >100 μg of *Eimeria* recombinant profilin protein in the absence of adjuvant provided protection against subsequent challenge infection with live parasites, whereas immunization with ≤50 μg of protein was inadequate to induce protective immunity (Ding et al., 2004). Therefore, we chose suboptimal vaccine doses ≤50 μg to evaluate the effect of profilin vaccination on the development of protective immunity in the absence or presence of ISA 71 VG. The main objective of this study was to evaluate the adjuvant activity of ISA 71 VG against two different species of *Eimeria* parasites, *E. acervulina* and *E. tenella*.

2. Materials and methods

2.1. Chickens

One-day-old male broiler chickens (Ross/Ross) were obtained from the Longenecker’s Hatchery (Elizabethtown, PA), housed in the Petersime starter brooder units, and provided with feed and water ad libitum. At 14 days post-hatch, the chickens were transferred to larger hanging cages housing two birds per cage and randomly divided into 11 groups (13 birds/group). All experiments were approved by the Beltsville Agricultural Research Center Small Animal Care and Use Committee.

2.2. Recombinant profilin protein

The 3-1E gene encoding recombinant profilin protein was originally derived from *E. acervulina* (EA) and expressed in *Escherichia coli* as described previously (Song et al., 2000; Lillehoj et al., 2005). The 1086-base pair cDNA was subcloned into the pMAL plasmid with an NH2-terminal maltose-binding protein epitope tag and a Factor Xa protease cleavage site between maltose-binding protein and profilin (Ding et al., 2004). Transformed E. coli DH5α bacteria were grown to mid-log phase, induced with 1.0 mM of isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, collected by centrifugation, and disrupted by sonication on ice (Misonix, Farmingdale, NY). The recombinant profilin protein was isolated on an amylose affinity column (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions, digested with Factor Xa to release profilin from the solid phase, and re-passed through the amylose column to remove any contaminating maltose-binding protein. Final purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting with profilin-specific rabbit antibody (Ding et al., 2004).

2.3. Adjuvant

Montanide™ ISA 71 VG (Seppic, Paris, France) is a ready-to-use blend of mineral oil and an ester of mannitol and oleic acid. Immunizing emulsion containing ISA 71 VG adjuvant was prepared by mixing adjuvant and profilin protein (in PBS) in the ratio 70:30 (w/w) and passing the suspension through the injection needle 0.9 G20 20 times.

2.4. Parasites

The strains of *E. acervulina* and *E. tenella* used in this study were originally developed and maintained at the Animal Parasitic Diseases Laboratory of the Animal and Natural Resources Institute (Beltsville, MD) (Song et al., 2000). Sporulated oocysts were cleaned by floatation on 2.5% sodium hypochlorite, washed three times with PBS, and enumerated using a hemocytometer before experimental infections.

2.5. Experimental design

The experimental plan is summarized in Table 1 and Fig. 1. At 1 week of age, chickens were subcutaneously immunized with 20, 30, or 40 μg of profilin emulsified in ISA 71 VG. Control chickens were immunized with PBS or with profilin in the absence of adjuvant. At 1 week post-primary immunization, chickens were given secondary subcutaneous booster injection identical with the primary immunization. At 7 days post-secondary immunization, all groups were orally challenged with 1.0 × 106 sporulated *E. acervulina* or *E. tenella* oocysts. PBS-injected birds which were uninfected remained as the unchallenged control group.

2.6. Body weight gains and fecal oocyst counts

Uninfected and coccidia-infected birds (*N* = 8/group) were assessed for body weight changes between 0 and 10 days post oral challenge infection with *E. acervulina* or *E. tenella* oocysts. For determination of fecal oocysts numbers, birds (*N* = 8/group) were placed on wire oocyst collection cages, fecal samples collected between 5 and 10 days post-challenge infection, and oocysts were individually enumerated using a McMaster counting chamber as described (Ding et al., 2004).

2.7. Serum anti-profilin antibody levels

At 7 days post-secondary immunization, birds (*N* = 5/group) were euthanized by cervical dislocation, blood was collected by cardiac puncture, sera were prepared by low speed centrifugation, and used to measure profilin-specific antibody responses by ELISA (Ding et al., 2004). Microtiter plates were coated overnight with 10 μg/well of purified recombinant profilin, washed with PBS containing 0.05% Tween 20, and blocked with PBS containing 1% BSA. Serum samples were incubated with continuous shaking, the plates were washed, and bound antibody was detected with peroxidase-conju-
gated rabbit anti-chicken IgG and peroxidase-substrate (Sigma, St. Louis, MO). OD values at 450 nm (OD\textsubscript{450}) were measured with an automated microplate reader (Bio-Rad, Richmond, CA).

2.8. Spleen lymphoproliferation

At 7 days post-secondary immunization, birds (\(N = 3\)/group) were euthanized by cervical dislocation and spleen single cell suspensions were prepared as described (Lee et al., 2007). Lymphocytes were adjusted to \(5.0 \times 10^6\) cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin in 96-well microtiter plates and unstimulated or stimulated with 20 \(\mu\)g/ml of profilin at 41 °C and 5% CO\textsubscript{2} for 48 h. As a positive control, the cells were stimulated with 5.0 \(\mu\)g/ml of concanavalin A (Con A). Cell proliferation was measured using WST-8 (Cell-Counting Kit-8, Dojindo Molecular Technologies, Gaithersburg, MD) at OD\textsubscript{450}. Lymphoproliferation was expressed as stimulation index (SI), which is the ratio of the mean optical density (OD) value of the Con A- or profilin-stimulated group divided by mean OD value of the medium-only stimulated control group.

2.9. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mean values of treatment groups were compared using the Duncan’s multiple range test and differences were considered statistically significant at \(P < 0.05\).

3. Results

3.1. Effect of vaccination with profilin plus ISA 71 VG on body weight gain

As shown in Fig. 2, birds immunized with profilin alone and infected with \textit{E. acervulina} or \textit{E. tenella} oocysts had reduced body weight gains between 0 and 10 days post-challenge infection compared with uninfected chickens. However, vaccination with 40 \(\mu\)g of profilin plus ISA 71 VG prior to parasite infection increased weight gains to the similar level seen in uninfected birds.

3.2. Effect of vaccination with profilin plus ISA 71 VG on fecal oocyst shedding

As shown in Fig. 3, vaccination with 30 or 40 \(\mu\)g of profilin plus ISA 71 VG was associated with decreased numbers of fecal oocysts in \textit{E. acervulina}- or \textit{E. tenella}-infected animals compared with birds immunized with profilin alone.
3.3. Effects of vaccination with profilin plus ISA 71 VG on profilin serum antibody responses

Serum antibody titers against profilin at 7 days post-secondary immunization were greater in all groups of birds vaccinated with profilin plus ISA 71 VG compared with birds given profilin alone (Fig. 4). In particular, antibody levels as measured by ELISA OD450 values were approximately 2-fold greater using 30 μg of profilin plus ISA 71 VG compared with the profilin-only control group.

3.4. Effect of vaccination with profilin plus ISA 71 VG on splenic lymphoproliferation

All experimental groups immunized with profilin plus ISA 71 VG exhibited increased both Con A- and profilin-stimulated splenocyte proliferation at 7 days post-secondary immunization compared with profilin-only immunized animals, with the 40 μg antigen plus the adjuvant group displaying the highest level of antigen-stimulated lymphoproliferation (Fig. 5).

4. Discussion

This study demonstrated that: (1) chickens vaccinated with 30 or 40 μg of profilin plus ISA 71 VG showed reduced fecal oocyst shedding following E. acervulina and E. tenella challenge infection compared with animals vaccinated with 50 μg of profilin without any adjuvant, and (2) the adjuvant–vaccine combination enhanced profilin-specific serum antibody levels and increased Con A- and profilin-stimulated lymphoproliferation in all groups compared with the vaccine-only group. We chose the lower doses of profilin in the presence of ISA 71 VG compared with the profilin-only group based on our previous observations using the ISA 70 VG formulation (Jang et al., 2010). In the current study, we asked whether ISA 71 VG was a more effective adjuvant compared with ISA 70 VG by using lower antigen doses. The current data suggest that ISA 71 VG not only stimulates more complete protection against experimental avian coccidiosis compared with ISA 70 VG, but also does so at lower vaccine doses. The ability of ISA 71 VG to increase Con A-induced splenocyte proliferation is an indication of its non-specific adjuvant effect on cell-mediated immunity in chickens.

The Montanide ISA series of W/O emulsion adjuvants have shown superior efficacy compared with traditional adjuvant formulations (aluminum hydroxide and oil-based emulsions) with a variety of human and animal vaccines (Bowersock and Martin, 1999; Gupta et al., 1995; Cox et al., 2003). Water-in-oil adjuvants based on squalene, such as ISA 720, have been tested in human clinical trials, and other studies have demonstrated their ability to stimulate protective immunity against HIV and malaria vaccines (Toledo et al., 2001; Aucouturier et al., 2006; Pattanaik et al., 2007). East et al. (1992) showed that Montanide™ ISA 50 was superior to aluminum hydroxide for vaccination of sheep with a partially purified extract of Lucilia cuprina (blowfly) larvae. Iyer et al. (2001) demonstrated that Montanide™ ISA 57 elicits early and long lasting antibody titers in foot-and-mouth disease-vaccinated guinea pigs. In chickens, vaccination against Newcastle disease virus in

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Fig. 3. Effect of vaccination with profilin plus ISA 71 VG on fecal oocyst shedding following oral challenge infection with E. acervulina or E. tenella. Birds were subcutaneously immunized with PBS, profilin alone (50 μg), or 20, 30, or 40 μg of profilin plus ISA 71 VG, as illustrated in Table 1, and fecal oocyst numbers were determined between 5 and 10 days post infection. Each bar represents the mean ± S.D. value (N = 8). Within each graph, bars with different letters are significantly different according to the Duncan’s multiple range test (P < 0.05).

Fig. 4. Effect of vaccination with profilin plus ISA 71 VG on profilin serum antibody responses. Birds were subcutaneously immunized with PBS, profilin alone (50 μg), or 20, 30, or 40 μg of profilin plus ISA 71 VG as illustrated in Table 1, and profilin serum antibody levels were determined by ELISA at 7 days post-secondary immunization. Each bar represents the mean ± S.D. value (N = 5). Bars with different letters are significantly different according to the Duncan’s multiple range test (P < 0.05).
combination with a mineral oil emulsion adjuvant increased protective immunity against subsequent challenge infection (Aucouturier and Ganne, 2000; Aucouturier et al., 2001). Apart from our prior study with the Eimeria profilin vaccine (Jang et al., 2010), however, Montanide type adjuvants have not previously been tested for their ability to augment immunity against avian coccidiosis.

Profilin is an actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton. It is found in all eukaryotic organisms in most cells. In apicomplexan protozoa, such as Eimeria, Plasmodium, and Toxoplasma, profilin is a key contributor to actin-dependent gliding motility that is essential for migration across biological barriers and host cell invasion (Yarovinsky et al., 2005). As a result, profilin has been considered as a vaccine candidate with the expectation that anti-profilin antibodies may block parasite invasion. In addition, profilin itself can exert an adjuvant function to stimulate cell-mediated immunity. For example, toxofilin, the profilin of Toxoplasma gondii, binds to Toll-like receptor (TLR)11, inducing a potent IL-12 response in dendritic cells, and consequently induced both enhanced specific humoral and Th1 cellular immune responses (Hedhli et al., 2009; Plattner et al., 2008; Yarovinsky et al., 2005). Eimeria profilin was identified in the merozoites of the Eimeria parasite as an immunogenic protein which induced antigen-specific proliferation and IFN-γ production by chicken splenic lymphocytes (Lillehoj et al., 2000). Subcutaneous immunization of broiler chickens with recombinant profilin expressed in E. coli, or in ovo vaccination of 18-day-old embryos with a DNA plasmid encoding the profilin gene, increased protective immunity to subsequent challenge infection by E. acervulina (Song et al., 2000; Lillehoj et al., 2000).

In this study, profilin-reactive antibodies and antigen-specific splenocyte proliferation were used as indicators of acquired immune immunity following experimental Eimeria infection. During natural infection with coccidia parasites, T-cell immunity, as opposed to humoral immunity, plays the dominant role in protection against infection (Lillehoj and Lillehoj, 2000). However, under some circumstances, such as in response to Eimeria subunit vaccination against immunodominant epitopes, antibody titers have been shown to be a good predictor of protective immunity (Lee et al., 2009). In the case of cell-mediated immune responses, presence of antigen-sensitized T cells and antigen-specific splenocyte proliferation is often used to assess the degree of immunity, and the levels of lymphocyte stimulation have been shown to directly correlate with the extent of disease protection (Lillehoj, 1986; Lillehoj et al., 1988).

Although cross-species protection does not occur in the field, Eimeria antigens capable of inducing cross-species immunity have been demonstrated using synthetic peptide motifs selected on the basis of immunogenic sequences conserved across different species (Talebi and Mulcahy, 2005). In theory, therefore, the ideal vaccine candidate should contain conserved immunodominant epitopes capable of generating cross-protection to multiple coccidia variants, strains, and species. In this regard, Crane et al. (1991) reported that chickens immunized with a recombinant antigen of E. tenella, CheY-So7, developed protective immunity against the homologous parasite as well as E. acervulina, E. maxima, and Eimeria necatrix. Ding et al. (2005) also showed that chickens immunized with a recombinant plasmid encoding an E. tenella microneme gene (MIC2) acquired cross-protection against challenge infection with E. acervulina, but not E. maxima. Finally, Xu and colleagues (2008) described two chimeric cross-protective recombinant DNA vaccines encoding Eimeria protein antigens linked to the chicken IL-2 cytokine. The first, pcDNA-TA4-IL-2, protected against E. tenella, E. necatrix, and E. acervulina and the second, pVAX1-cSZ2-IL-2, protected against E. tenella, E. necatrix and E. maxima (Song et al., 2009; Shah et al., 2010). To date, however, no reports have described a single vaccine or immunization protocol that generates protective immunity against all seven species of Eimeria that infect chickens. Future studies to identify other relevant coccidia antigens and antigen-adjuvant combinations which are capable of inducing cross-immunity to coccidiosis of poultry will be important milestones in the quest to obtain an effective non-drug control strategy against this disease.

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