Mucosal immunity against *Eimeria acervulina* infection in broiler chickens following oral immunization with profilin in Montanide™ adjuvants

Seung I. Jang a,1, Hyun S. Lillehoj a,e, Sung Hyen Lee a, Kyung Woo Lee a, Erik P. Lillehoj b, François Bertrand c, Laurent Dupuis c, Sébastien Deville c

aAnimal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, USA
bDepartment of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, USA
cSEPPIC, 22 Terrasse Bellini, 92800 Puteaux, France

doi:10.1016/j.exppara.2011.05.021

0014-4894/$ - see front matter Published by Elsevier Inc.

1. Introduction

Coccidiosis is the name given to a group of closely related diseases caused by a single-celled intestinal protozoan parasite, *Eimeria*. Intestinal infection of chickens by *Eimeria* reduces feed utilization, leading primarily to reduced body weight gain and secondarily to mortality. Avian coccidiosis causes more than $3 billion annual losses globally in the poultry industry (Lillehoj et al., 2000). Traditionally, prophylactic medications have been used to control avian coccidiosis, but novel approaches are urgently needed due to the appearance of drug-resistant coccidia parasites and increasing regulatory ban of antibiotic use in commercial poultry production (Chapman, 1997; Williams, 2002). Recombinant *Eimeria* protein subunit vaccines offer a means to elicit antigen-specific protective immunity against coccidiosis, and further enhancement of immunity can be achieved using protein vaccines in conjunction with adjuvants (Lillehoj et al., 2005; Jang et al., 2010, 2011).

Incomplete and complete Freund’s adjuvants, lipid A derivatives, Quil A, and the Montanide™ ISA and IMS series of adjuvants have been used to improve vaccine potency in a variety of animal systems (Lacaille-Dubois and Wagner, 1996; Oda et al., 2004; Jang et al., 2010, 2011). Among the most recently developed, the Montanide™ ISA and IMS series of adjuvants have proven to be safe and efficacious in association with bacterial, viral, and parasitic vaccines (Cauchard et al., 2004; Belloc et al., 2008; Jarvi et al., 2008; Jang et al., 2010). For example, Montanide™ ISA 71 VG is a water-in-oil emulsion, and IMS 1313 N VG PR is an aqueous-based nanoparticle adjuvant, both of which have generated superior immunostimulating activity against...
veterinary vaccines, such as those against foot and mouth disease and Rhodococcus equi (Barnett et al., 1996; Aucouturier et al., 2001, 2006; Cloete et al., 2008).

In a previous report, we described that vaccination with an Eimeria recombinant profilin protein plus the Montanide™ ISA 71 VG adjuvant increased protection against experimental avian coccidiosis (Jang et al., 2010). Further, this antigen/adjuvant combination amplified cross-protection against non-immunizing Eimeria spp. (Jang et al., 2011). While water-in-oil adjuvants are valuable means of increasing immunogenicity under experimental conditions, vaccine delivery to mucosal surfaces under field conditions is generally more effective using aqueous solutions. Therefore, the current investigation was undertaken to compare profilin plus ISA 71 or IMS 1313 adjuvants for enhancing protection against experimental Eimeria infection.

2. Materials and methods

2.1. Animals

One day-old male broiler chickens (Ross strain, Longenecker’s Hatchery, Elizabethtown, PA) were reared in Petersime starter brooder units and provided with feed and water ad libitum. At 14 days post-hatch, the chickens were transferred to hanging cages with two birds per cage. All procedures were approved by the Beltsville Area Institutional Animal Care and Use Committee.

2.2. Recombinant profilin protein and commercial vaccine

Eimeria recombinant profilin protein was expressed in Escherichia coli and purified as described (Jang et al., 2011). The commercial coccidiosis vaccine, Coccivac-B (Intervet/Schering-Plough, Millsboro, DE), is a mixture of live Eimeria acervulina, Eimeria mivati, Eimeria maxima, and Eimeria tenella oocysts.

2.3. Adjuvants and vaccine formulation

Montanide™ ISA 71 VG (ISA 71; SEPPIC, France) is a ready-to-use water-in-oil emulsion. Montanide™ IMS 1313 N VG PR (IMS 1313; SEPPIC, France) is a dispersion of liquid nanoparticles in an aqueous phase containing an immunostimulating component and compatible with a wide variety of vaccine antigens. Profilin was mixed with ISA 71 at a 30:70 ratio (wt.:wt., profilin:adjuvant) or with IMS 1313 at a 50:50 ratio as recommended by the manufacturer.

2.4. Parasite

The strain of E. acervulina used in this study was originally developed and maintained at the Animal Parasitic Diseases Laboratory of the Animal and Natural Resources Institute (Beltsville, MD) (Jang et al., 2011). Oocysts were cleaned by flotation on 2.5% sodium hypochlorite, washed three times with PBS, and enumerated using a hemocytometer prior to experimental infections.

2.5. Immunization

The experimental plan is summarized in Table 1 and Fig. 1. Birds were randomly divided into six groups (20 birds/group). For the Coccivac-B group, 1 day-old chickens were ocularly vaccinated as recommended by the manufacturer. For the profilin-only or profilin/adjuvant groups, 7 day-old chickens were subcutaneously immunized with 50 µg of recombinant profilin with or without ISA 71, or orally immunized with 50 µg of profilin with IMS 1313. Unimmunized chickens received PBS. At 7 days post-primary immunization, secondary immunizations were given with PBS, 50 µg of profilin, or 50 µg of profilin/adjuvant mixtures. At 7 days post-secondary immunization, all groups were orally infected with 1.0 × 10⁴ sporulated E. acervulina oocysts. Non-infected and PBS-injected birds were used as the unchallenged controls.

2.6. Body weight gain and fecal oocyst shedding

Uninfected and Eimeria-infected birds (8/group) were assessed for body weight gain between 0 (21 day-old) and 10 (31 day-old) days post-infection. Fecal samples were collected from infected birds between 6 and 10 days post-infection and oocysts were enumerated using a McMaster counting chamber as described (Ding et al., 2004).

2.7. Intestinal secretory sIgA and IgY levels

At 7, 10, and 14 days post-secondary immunization, chickens were killed by cervical dislocation, and intestines were removed, cut longitudinally, and incubated for 4 h on ice in 10 ml of ice-cold PBS containing 0.05 trypsin inhibitory units/ml of aprotinin, 5.0 mM EDTA, 2.0 mM phenylmethylsulfonyl fluoride, and 0.02% NaN₃ (Sigma, St. Louis, MO). Ninety-six-well microtiter plates were coated overnight with 1.0 µg/well of profilin. The plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with PBS containing 1.0% BSA. Intestinal washes were added (100 µl/well), incubated with constant agitation for 2 h at room temperature, and washed with PBS-T. Bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgA or IgY antibodies and 3,3',5,5'-tetramethylbenzidine substrate (Sigma). Optical density at 450 nm (OD₄₅₀) was measured with an automated microplate reader (Bio-Rad, Richmond, CA). All samples were analyzed in triplicate.

2.8. Intestinal intraepithelial lymphocyte (IEL) subpopulations

At 7 and 14 days post-secondary immunization, intestinal sections from the duodenum to the ileum were removed, cut longitudinally, and washed with ice-cold Hank’s balanced salt solution without calcium chloride and magnesium sulphate (HBSS, Sigma). Tissue sections were treated for 20 min at 37 °C with HBSS containing 10 mM dithiothreitol (Sigma), and 0.1 mM EDTA with continuous shaking. Released cells were passed through nylon wool and IELs were purified by density gradient centrifugation as described (Min et al., 2001). IELs were resuspended in HBSS containing 3.0% FBS and 0.01% NaN₃, incubated with mouse monoclonal antibodies against chicken CD4⁺, CD8⁺, TCR1⁺ or TCR2⁺, respectively, and analyzed by flow cytometry (BD FACSAria II, BD Biosciences, San Jose, CA) as described (Min et al., 2001).

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. Vaccination with profilin plus ISA 71 increases post-infection body weight gain

Chickens immunized with profilin plus ISA 71, but not IMS 1313, exhibited increased body weight gain compared with animals vaccinated with profilin alone (P < 0.05; Fig. 2A). Weight gain of the profilin/ISA 71 group was equal to that of chickens given the Coccivac vaccine.

3.2. Vaccination with profilin plus ISA 71 or IMS 1313 decreases post-infection oocyst shedding

Chickens immunized subcutaneously with profilin plus ISA 71, or orally with profilin plus IMS 1313, had decreased fecal oocyst shedding compared with birds vaccinated with profilin alone (P < 0.05; Fig. 2B). IMS 1313 was more effective than ISA 71 in reducing oocyst shedding. Fecal parasite numbers in Coccivac-B vaccine group were decreased the furthest.

3.3. Vaccination with profilin plus ISA 71 or IMS 1313 increases intestinal sIgA and IgY parasite antibody levels

Intestinal levels of sIgA antibodies against profilin at 7, 10, and 14 days post-secondary immunization were increased in chickens vaccinated with profilin plus ISA 71 or IMS 1313 compared with chickens given profilin alone (P < 0.05; Fig. 3A). By contrast, profilin plus ISA 71 was more effective in enhancing profilin-reactive IgY levels compared with profilin alone or profilin plus IMS 1313, particularly at 7 and 10 days post-secondary vaccination (P < 0.05; Fig. 3B). Both sIgA and IgY profilin antibody levels were greater in Coccivac-B-vaccinated chickens compared with unvaccinated controls.

3.4. Vaccination with profilin plus ISA 71 or IMS 1313 increases intestinal CD4+, CD8+, and TCR2+ IEL subpopulations

The percentages of CD4+, CD8+, TCR1+, and TCR2+ intestinal IELs were determined at 7 days post-secondary vaccination and 7 days post-infection (14 days post-secondary vaccination). Immunization with profilin plus ISA 71 or IMS 1313 increased intestinal CD4+ and CD8+ IELs at both time points compared with the profilin-only group (P < 0.05; Fig. 4A and B). Both profilin/adjuvant groups also had greater TCR1+ cells at 7 days post-secondary vaccination and more TCR2+ IELs at 14 days post-secondary immunization, compared with the profilin-only group (P < 0.05; Fig. 4C and D). In addition, the profilin/ISA 71 group had increased TCR2+ IELs at 7 days post-secondary immunization. Comparison of the profilin/adjuvant groups with the Coccivac-B group revealed that the former exhibited greater CD4+ IELs at both time points, while the latter had greater TCR1+ cells at 14 days post-secondary vaccination.

4. Discussion

The major findings of this paper are that profilin/IMS 1313- or profilin/ISA 71-immunized chickens had decreased fecal oocyst excretion, increased intestinal sIgA antibody levels against profilin, and increased percentages of CD4+, CD8+, and TCR2+ intestinal IELs compared with birds vaccinated with profilin alone. Profilin plus IMS 1313 was superior to profilin with ISA 71 for decreasing parasite shedding and increasing CD8+ IELs. Profilin with ISA 71 was better than profilin plus IMS 1313 for enhancing body weight gain and increasing profilin-reactive IgY antibody levels. By comparison, the Coccivac-B- and profilin/ISA 71-vaccinated chickens had similar profiles of improvement in disease parameters vs. their respective controls, with the exception of CD4+ IELs which were greater in the latter. These results confirm and extend our prior studies demonstrating the utility of Montanide™ adjuvants in controlling experimental E. acervulina infection (Jang et al., 2010, 2011).

In general, vaccination strategies using protein-, peptide-, or DNA-derived pathogen components are less effective at inducing protective immunity against infectious pathogens compared with the whole microorganisms (Watts and Kennedy, 1999). In the case of avian coccidiosis, the efficacy of recombinant antigen vaccination has been limited, in large part, due to not only the restricted expression of coccidia proteins during the different phases of the parasite life cycle, but also lack of strong immunological cross-reaction among the multiple Eimeria species (Lillehoj et al., 2000; Song et al., 2000; Ding et al., 2004; Lillehoj et al., 2005). The current study, and others (Barnett et al., 1996; Belloc et al., 2008), indicate
that combination of restricted and/or weak pathogen antigens with novel, second-generation adjuvants provides a sufficient level of immunostimulation to increase protective immunity against challenge infection under experimental conditions. Whether these, or other, adjuvants also will be effective in field trials remains to be established.

In addition to innovative immunomodulators, the route of vaccination greatly influences the development of immunity against human and veterinary pathogens (Vyas and Gupta, 2007). Mucosal vaccination via the oral or ocular routes normally favors the generation of neutralizing sIgA antibodies and protective cell-mediated immune responses (Wang et al., 2004). Earlier studies showed that following *Eimeria* infection, chickens produce profilin-specific serum IgY and IgA antibodies that generally reached peak levels between 7 and 20 days post-infection (Trees et al., 1989; Yun et al., 2000). Increased levels of sIgA or IgY antibodies in the gut also were seen in chickens immunized with a profilin/adjuvant complex (Lillehoj et al., 2004; Lee et al., 2009). However, the mucosal route of delivery has practical limitations, including low pH, gastric proteolytic enzymes, rapid transit through the intestine, and poor absorption of large molecules. Accordingly, nanoparticle-based adjuvants have been engineered to protect antigens in the gut, to target antigens to the gut-associated lymphoid tissues (GALT), and to increase the residence time of the antigen in the intestine through their adhesive properties.

Chicken intestinal IELs play a critical role in the generation of a complex immunoregulatory network during *Eimeria* infection and the ensuing host response (Lillehoj and Trout, 1996). This network is initiated and maintained through the local production of pro- and anti-inflammatory cytokines and chemokines. In a previous study, we found that broiler chickens immunized with profilin plus ISA adjuvants had altered levels in the gut of gene transcripts encoding interleukin (IL)-2, IL-10, IL-17A, and interferon (IFN)-γ (Jang et al., 2010). Among these, IFN-γ expression was directly correlated with protective cell-mediated immunity against avian coccidiosis mediated by CD4+ and CD8+ effector lymphocytes (Choi et al., 1999; Yun et al., 2000; Cornelissen et al., 2009). On the basis of these prior reports and the current results, we speculate that vaccination with profilin plus ISA 71 or IMS 1313 stimulates the recruitment of CD4+ and/or CD8+ cells to the intestine where they mediate a protective, IFN-γ-dependent anti-coccidial response. Current studies are ongoing to test this hypothesis.
In conclusion, these results provide evidence that experimental immunization of chickens with *Eimeria* profilin in combination with Montanide™ ISA 71 VG or IMS 1313 N VG PR adjuvants increases protection against infection by *E. acervulina*. The precise molecular and cellular nature of the protective host responses induced by these complexes remains to be determined.

Acknowledgments

This project was partially supported by a Trust agreement established between ARS, USDA and SEPPIC, Inc. (Puteaux, France) and The World Class University Program (R33-10013) of The Ministry of Education, Science and Technology of South Korea. The authors thank Ms. Margie Nichols and Ms. Stacy Torreysen for their technical assistance.

References


