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Incorporation of a recombinant *Eimeria maxima* IMP1 antigen into nanoparticles confers protective immunity against *E. Maxima* challenge infection

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ABSTRACT

The purpose of this study was to determine if conjugating a recombinant *Eimeria maxima* protein, namely EmaxIMP1, into 20 nm polystyrene nanoparticles (NP) could improve the level of protective immunity against *E. maxima* challenge infection. Recombinant EmaxIMP1 was expressed in *Escherichia coli* as a poly-His fusion protein, purified by NiNTA chromatography, and conjugated to 20 nm polystyrene NP (NP-EmaxIMP1). NP-EMaxIMP1 or control non-recombinant (NP-NR) protein were delivered *per os* to newly-hatched broiler chicks with subsequent booster immunizations at 3 and 21 days of age. In battery cage studies (n = 4), chickens immunized with NP-EMaxIMP1 displayed complete protection as measured by weight gain (WG) against *E. maxima* challenge compared to chickens immunized with NP-NR. WG in the NP-EMaxIMP1-immunized groups was identical to WG in chickens that were not infected with *E. maxima* infected chickens. In floor pen studies (n = 2), chickens immunized with NP-EMaxIMP1 displayed partial protection as measured by WG against *E. maxima* challenge compared to chickens immunized with NP-NR. In order to understand the basis for immune stimulation, newly-hatched chicks were inoculated *per os* with NP-EMaxIMP1 or NP-NR protein, and the small intestine, bursa, and spleen, were examined for NP localization at 1 h and 6 h post-inoculation. Within 1 h, both NP-EMaxIMP1 and NP-NR were observed in all 3 tissues. An increase was observed in the level of NP-EmaxIMP1 and NP-NR in all tissues at 6 h post-inoculation. These data indicate that 20 nm NP-EmaxIMP1 or NP-NR reached deeper tissues within hours of oral inoculation and elicited complete to partial immunity against *E. maxima* challenge infection.

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1. Introduction

Considerable research over the last decade has shown the efficacy of nanoparticles (NP) to deliver drugs and vaccines for treating a variety of diseases (for review see [1–4]). NP have been found to be readily taken up by antigen-presenting cells and stimulate different arms of the immune system including helper T cells, with subsequent cytokine release [2]. NP smaller than 50 nm are efficiently internalized at mucosal surfaces (5,6), and when conjugated to a protein antigen, induce both mucosal and systemic immune responses following mucosal administration [6]. In addition, NP-conjugated recombinant proteins have been used to elicit protective immunity against a number of parasitic diseases including malaria [7–11], leishmaniasis [12–15], and toxoplasmosis [16–18].

Avian coccidiosis is generally considered the most important disease affecting weight gain and feed utilization efficiency in commercial broilers. The causative organisms, protozoa in the genus *Eimeria*, invade and undergo rapid development in specific regions of the gut and thus disrupt nutrient uptake. Of equal importance is that *Eimeria* infection, particularly with *E. maxima*, predisposes chickens to colonization of the affected intestinal region by *Clostridium perfringens* which can lead to necrotic enteritis and increased mortality. Numerous recombinant *Eimeria* antigens have been shown to confer protective immunity against avian

Abbreviations: NP, nanoparticle; EmaxIMP1, recombinant *Eimeria maxima* immune-mapped protein 1; NR, non-recombinant; NINC, non-immunized non-challenged; NIC, non-immunized challenged.

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coccidiosis (for review see [19]), which has generally been measured by a decrease in parasite development (oocyst excretion) at relatively low challenge doses. Of the recombinant *Eimeria* antigens showing protective efficacy, the *E. maxima* immune-mapped protein 1 (EmaxIMP1) appears to be a promising candidate for inclusion in a subunit vaccine against coccidiosis [20]. Our preliminary studies using recombinant EmaxIMP1 delivered by intramuscular, intranasal, or oral inoculation has failed to provide significant protection against clinical effects of *E. maxima* challenge (unpublished observations). The purpose of this study was to determine whether delivering recombinant EmaxIMP1 as a conjugate to 20 nm NP could improve the level of protection against *E. maxima* infection as measured by weight gain, arguably one of the most important clinical parameters associated with avian coccidiosis.

2. Materials and methods

2.1. Preparation of recombinant EmaxIMP1 conjugated to NPs

Recombinant EmaxIMaxIMP1 was expressed as a polyHis fusion protein in *Escherichia coli* BL21 (from the pTrcHis expression vector, Invitrogen, Carlsbad, CA) or *E. coli* Top10 (from the pBadHis expression vector, Invitrogen) as described elsewhere [21], and purified by NiNTA affinity chromatography following manufacturer's instructions (Invitrogen). The purity of EmaxIMP1 in NiNTA eluates was confirmed by SDS-PAGE followed by staining with colloidal Coomassie blue staining; the concentration of EMaxIMP1 protein was determined by BCA assay (Pierce Chemical Co., Dallas, TX). NP-EmaxIMP1 were prepared using a procedure described elsewhere [5]. An equivalent amount of NR protein was conjugated to NP in a separate tube. After NP conjugation, the mixture was dialyzed against 0.01 M PBS (pH 7.4) for 3 days at 4 °C using a Float-A-Lyzer membrane (100 kDa cutoff) (Spectrum Labs, Inc., Rancho Dominguez, CA) with 2 changes of PBS each day. After dialysis, the NP-EmaxIMP1 and NP-NR were transferred to a 1.5 ml microcentrifuge tube and diluted with PBS to achieve a 20% NP solution. The conjugation of EMaxIMP1 to NP was confirmed by dot blot analysis using rabbit anti-EMaxIMP1 sera followed by FITC-conjugated anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) as described elsewhere [21].

2.2. Tissue localization of NP-EmaxIMP1 and NP-NR following per os administration

Newly-hatched male broiler chicks (Longeneckers Hatchery, Elizabethtown, PA) were inoculated *per os* with 100 μl of NP-EmaxIMP1 or NP-NR. After 1 h or 6 h, the chicks (n = 3/timepoint and treatment) were euthanized for necropsy to remove the small intestine, bursa, and spleen. For the small intestine, a 2 cm section flanking 1 cm on either side of the Meckel's diverticulum was excised, and the intestinal lumen was gently filled with Tissue-Tek OCT Compound (Sakura, Torrance, CA) using a syringe with a blunt-end needle. The intestine was then placed in a Tissue-Tek vinyl Cryomold (25 mm × 20 mm × 5 mm, Sakura) filled about half-way with OCT. The tissue-containing Cryomold was placed on a block of dry ice and OCT was immediately added to the top of the Cryomold. After the block was completely frozen, the Cryomold was placed in a separate dry ice container. A similar procedure was followed for bursa and spleen with some modifications. For bursa, a longitudinal cut was made to divide the tissue in half. One half of the bursa was left intact, while individual plicae were separated using a scapel; all sections were then placed in a single Cryomold containing OCT compound and processed as described above. The spleen was excised, excess fat removed, and the entire tissue was transferred to a single Cryomold containing OCT compound and processed as described above.

2.2.1. Parasites

Coccidiosis challenge infection utilized *Eimeria maxima* (APU1) oocysts that had been propagated in susceptible chickens every 3–4 months for 10 years after initial isolation [22]. The purity of *E. maxima* oocysts was confirmed by ITS1-PCR using procedures described elsewhere [23].

2.3. Battery cage studies

Newly hatched (<6 h-old) broiler chicks (n = 3 replicates/treatment, 4 chicks/replicate; male Hubbard/Ross HR708, Longeneckers Hatchery) were inoculated *per os* with 40 μl NP-EmaxIMP1 or NP-NR. On days 3 and 21, all vaccinated chicks received an oral booster immunization with 40 μl of the identical NP immunogen used in the primary immunization. All chicks, including non-immunized controls, were housed by treatment groups in separate cages of a Petersime starter unit (Petersime, Gettysburg, OH), and transferred at 2 wk of age to individual cages of Petersime finisher units at 3 cages/treatment and 4 chickens/cage. Chicks were provided water and standard poultry ration (crumbles, 24% protein) *ad libitum*. At 4 wk of age, all immunized chickens, and a control group of non-immunized chickens (NIC), were challenged with 750 *E. maxima* (APU1) oocysts. This challenge dose was based on dose titration studies designed to achieve a 20–25% reduction in weight gain in non-immunized challenged (NIC) controls. Another group of non-immunized chickens were not challenged with *E. maxima* oocysts to serve as non-challenged controls (NINC). Body weights of all chickens were measured on day of challenge infection and 6 days post-challenge in order to calculate weight gain during the peak infection period. Feed conversion ratio (FCR) was also calculated for each replicate by dividing average feed consumption by average weight gain during the infection period. A total of 4 separate vaccination-challenge battery cage studies were conducted to evaluate the protective effect of NP-EMaxIMP1 against *E. maxima* infection.

2.4. Floor pen studies

Newly hatched (<6 h old) broiler chicks (n = 3 replicates/treatment, 10 chicks/replicate; male Hubbard/Ross HR708, Longeneckers Hatchery) were inoculated *per os* with 40 μl NP-EmaxIMP1 or NP-NR. On days 3 and 21, all vaccinated chicks received an oral booster immunization with 40 μl of the identical NP-antigen used in the primary immunization. All chicks in each treatment group, including non-immunized controls, were assigned to 3 separate pens (10 chicks/pen), each pen measuring 1.5 m × 0.5 m × 0.75 m (length × width × height). The bottom of each pen contained 5–10 cm in depth wood shavings, and each pen was raised about 0.5 m above a concrete floor. Chicks were provided water and standard poultry ration (crumbles, 24% protein) poultry feed *ad libitum*. At 4 wk of age, all immunized chickens, and a control group of non-immunized chickens (NIC), were challenged with 750 *E. maxima* (APU1) oocysts. Another group of non-immunized chickens were not challenged with *E. maxima* oocysts to serve as non-challenged controls (NINC). Body weights of all chickens were measured on day of *E. maxima* challenge and 6 days post-challenge in order to calculate weight gain during the peak infection period. FCR was also calculated for each replicate by dividing average feed consumption by average weight gain during the infection period. A total of 2 separate vaccination-challenge floor pen studies were conducted to evaluate the protective effect of NP-EMaxIMP1 against *E. maxima* infection.