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Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with *Clostridium perfringens*

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Abstract

Necrotic enteritis (NE) in poultry has re-emerged as a concern for poultry producers, due in part to banning, by many countries, of the use of antimicrobial growth promoters in feeds. This re-emergence has led to a search for alternative methods for control of the disease, particularly vaccination. The objective of this work was to determine if vaccination of broiler chicks with recombinant alpha toxin protected against experimental challenge. Broiler chicks were vaccinated subcutaneously at 5 and 15 days of age, followed 10 days later by challenge with *Clostridium perfringens*. Birds were challenged twice daily on 4 consecutive days by mixing *C. perfringens* cultures with feed (three parts culture: four parts feed). Non-vaccinated birds challenged with *C. perfringens* developed NE at the rate of 87.8%, while only 54.9% of vaccinated birds developed lesions. In addition, non-vaccinated birds had lesion scores averaging 2.37, while average scores in vaccinated birds were 1.35. Vaccination produced an antibody response, with post-vaccination anti-alpha toxin IgG (IgY) titers in vaccinated birds more than 5-fold greater than in non-vaccinated birds. After challenge, vaccinated birds had average IgG (IgY) titers >15-fold higher than those in non-vaccinated birds. These results suggest that alpha toxin may serve as an effective immunogen, and, as such, may play a role in pathogenesis.

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Keywords: *Clostridium perfringens*; Poultry; Alpha toxin; Necrotic enteritis; Vaccination

1. Introduction

Prevention of food animal diseases has commonly been accomplished by administration of antimicrobials

in feed or water. However, there are rising concerns about effects on human health of such routine use of antimicrobials, particularly as this pertains to the transfer of antimicrobial resistance from animal pathogens to human pathogens (Hampson and Murdoch, 2003). The European Union has banned many antimicrobial growth promoters (AGPs), and the bans have been accompanied by increased incidence of

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poultry necrotic enteritis (NE) (Casewell et al., 2003; Lovland et al., 2003; Van Immerseel et al., 2004). The annual cost of necrotic enteritis to the poultry industry worldwide is estimated at US\$ 2 billion (McReynolds et al., 2004).

NE is most commonly caused by *Clostridium perfringens* type A (Al-Sheikhly and Truscott, 1977; Kaldhusdal et al., 2001; Lovland et al., 2004). The only so-called major toxin produced by toxinotype A is alpha toxin (CPA) (Hofshagen and Stenwig, 1992; Nauerby et al., 2003; Songer, 1996; Yoo et al., 1997). There has been considerable sentiment, although relatively little evidence, that CPA plays an important role in pathogenesis of NE. CPA induces mucosal damage in chicken intestinal loops (Fernandez Miyakawa and Uzal, 2005); crude toxin preparations reportedly produce lesions compatible with NE in germ-free chickens, an effect which is neutralized by anti-CPA serum (Fukata et al., 1988; Lovland et al., 2004). Recently, Keyburn et al. (2006) reported that conventional birds challenged with a CPA mutant developed lesions typical of NE, and they concluded that CPA is not involved in pathogenesis.

A few investigators have examined the role of anti-CPA immunity in protection against NE. Vaccination of broiler breeder hens with type A toxoids yielded anti-CPA antibodies which were passed to progeny and yielded partial protection against subclinical NE (Lovland et al., 2004). In another study, birds inoculated with a CPA mutant were subsequently protected against challenge with a virulent isolate (Thompson et al., 2006). The goal of the work described here was to investigate the value of recombinant CPA (rCPA) as a parenteral immunogen against NE. Results indicate that vaccination of broiler chicks with HIS-tagged rCPA provides partial protection against experimental challenge.

2. Methods and materials

2.1. Birds and care

Commercial jumbo Cornish × Rock female broiler chicks were obtained as 1-day old hatchlings from Murray McMurray Hatchery (Webster City, IA, USA). Birds were housed in 1.5 m diameter brooders constructed of 3 mm thick pegboard and divided into

three equal parts. Commercial wood shavings provided as bedding were replaced at 2-week intervals.

Birds were fed a commercial chick starter ration (20% protein, Eagle Milling, Casa Grande, AZ, USA) through experimental day 7. Thereafter, birds were fed a high protein feed (28% protein) mixed 50:50 with menhaden fishmeal (SeaLac, Omega Protein, Houston, TX, USA). On day 24, feed was withheld for 20 h, and birds were then challenged with *C. perfringens*. Water was available *ab lib* from galvanized steel automatic waterers.

2.2. Production of histidine-tagged CPA and vaccination

The entire *cpa* gene was cloned via standard methods into plasmid pTrcHisB (Invitrogen, Carlsbad, CA, USA), generating plasmid pJGS211. This construct was used to transform *E. coli* DH5 α , yielding strain JGS2445. This strain expressed *cpa* in its entirety, producing CPA with an N-terminal hexahistidine tag (HIS-CPA). JGS2445 was cultivated in 50 ml of LB broth with 100 μ g ampicillin per ml, incubated with shaking (200 rpm) at 37 °C overnight. LB overnight culture was diluted 1–20 in 500 ml LB broth with ampicillin (100 μ g/ml) and, incubated with shaking (200 rpm) at 37 °C to an OD₆₀₀ of 0.6. It was then induced with 2.5 ml of isopropyl-beta-D-thiogalactopyranoside (IPTG, Gold Biotechnology, St. Louis, MO, USA; 500 mM) and incubated for a further 3 h, after which cells were harvested by centrifugation (16,270 × g, 10 min) and re-suspended in binding buffer [20 mM Tris, 100 mM NaCl (pH 8.0)]. Cells were disrupted in a French pressure cell (2×) and the resulting solution centrifuged (26,890 × g, 15 min). DNase (160 U, Promega, Madison, WI, USA) was added to the supernatant fluid (~40 ml) and incubated at 37 °C for 15 min, then placed on ice. Recombinant CPA was then purified on TALON resin (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions. Bound proteins were eluted with imidazole (50 mM, pH 8.0) and 1.5 ml fractions were collected. An aliquot of each fraction was electrophoresed in a 10% SDS-PAGE gel to assess purity of the HIS-CPA, which appeared as a band at ~48 kDa. Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL, USA).

Birds were vaccinated subcutaneously (SQ) with 20 μg of HIS-CPA, emulsified 1:1 (w/v) with a proprietary Quil A-based adjuvant (Benchmark BioLabs, Lincoln, NE, USA). Vaccinations were administered at 5 and 15 days of age. No local or general side effects were observed in any of the vaccinated birds in any of the studies. Serum was collected from birds on arrival, prior to challenge (at 24 days of age), and at necropsy (on day 29).

2.3. Challenge inoculums and protocol

C. perfringens type A challenge strain JGS4143 (*netB*⁺; Keyburn et al., 2008), obtained from a chicken with NE, was stored at -80°C in 50% glycerol: 50% brain heart infusion (BHI; Difco, Detroit, MI, USA). For inoculum preparation, JGS4143 was streaked for isolation on BHI agar with 5% citrated bovine blood. After incubation under anaerobic conditions (5% H_2 : 5% CO_2 : 90% N_2) at 37°C for 24 h, 1–2 colonies were transferred into 10 ml cooked meat medium (CMM; Difco) in a Hungate tube and incubated in the same atmosphere at 37°C for 18 h. The resulting culture was used to inoculate 100 ml fluid thioglycollate broth (FTG; Difco), 10 ml of which was used to inoculate 100 ml CMM. Each culture was incubated for 18 h at 37°C prior to passage. Thirty-three millilitres of the CMM culture were used as inoculum for 1 l FTG medium. After 18 h incubation, this FTG culture was mixed with feed for challenge. A separate, serially passed culture was prepared for each challenge feeding (total $n = 8$). Sub-culturing method used for preparing inoculums has shown to be most effective at reproducing disease, and studies in our laboratory to simplify method have failed to produce disease. Numbers of colony-forming units (cfus) were determined by plating serial 10-fold dilutions on BHI agar.

Birds were inoculated on days 25–28. High protein feed and FTG medium culture were mixed in a ratio of 3:4 (v/v). The mixture, which had a paste-like consistency, was then placed in galvanized steel feed trays and offered to birds. Trays were cleaned and any remaining feed disposed of prior to each subsequent feeding. Water was available *ab lib* throughout the challenge period. Negative control birds were challenged with uninoculated FTG mixed with high protein feed at the same ratio.

On day 29, birds were euthanized by CO_2 asphyxiation. Necrotic intestinal lesions were scored (0: no gross lesions; 1+: thin-walled or friable small intestine; 2+: focal necrosis or ulceration; 3+: large patches of necrosis; 4+: severe or extensive necrosis typical of field cases) and segments fixed in 10% buffered formalin for histological examination.

2.4. Detection of anti-CPA antibodies

Wells of microtitration plates (Immulon 4 HBX microtiter plates; Thermo Scientific, Waltham, MA, USA) were coated with HIS-CPA by addition of 100 μl of 0.06 M carbonate buffer (0.018 M Na_2CO_3 : 0.045 M NaHCO_3 , pH 9.6) containing 1 ng antigen. Plates were incubated overnight at 4°C and washed twice with phosphate buffered saline (pH 7.2) with 0.3% Tween 20 (ICN Biomedicals, Solon, OH, USA) (PBS-T). Bovine serum albumin (BSA; 1%) in PBS-T (200 μl) was then added to each well and the plate incubated for 1 h at 4°C . Plates were again washed with PBS-T, and then 100 μl of 2-fold serial dilutions (1:10–1:10,240) of test sera added. After incubation for 1 h at 37°C , plates were washed nine times with PBS-T, 100 μl of goat anti-chicken IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD, USA; diluted 1:8000) were added, and plates were incubated 1 h at 37°C . After washing six times with PBS-T, 100 μl of *o*-phenylenediamine (OPD) substrate (200 $\mu\text{g}/\text{ml}$) (Sigma–Aldrich, St. Louis, MO, USA) was added and the plate incubated for 30 min at room temperature in the dark. The reaction was stopped with 50 μl of 3 M HCl and color development allowed to continue for an additional 15 min. Results were read spectrophotometrically at 490 nm. End-point ELISA titers were reported as the reciprocal of the last dilution which had an $\text{OD}_{490} \geq 0.4$. All assays were performed in duplicate, and any assays performed at different times with one dilution factor variation were repeated to confirm results.

2.5. Statistical analysis

A Pearson Chi-square test was used for statistical comparison of rates of lesion development, rates of severe lesions and mortality across treatment groups.

Table 1
Response of vaccinated birds and controls to challenge with *Clostridium perfringens*

Treatment	Number of birds with severe lesions (score 3 or 4) ^{a,b}	Number of birds with gross lesions (%) ^{a,b}	Average lesion score ^{a,b}
rCPA vaccination, challenged	9/51 (17.6%)	28/51 (54.9%)	1.31 ± 0.17
Adjuvant only, challenged	20/49 (40.8%)	43/49 (87.8%)	2.37 ± 0.18
Non-vaccinated, non-challenged	0/48 (0.0%)	0/48 (0.0%)	0.00 ± 0.00
<i>p</i> value	0.0095	0.0001	0.0001

^a Composite of three independent studies.

^b Average of 16 birds per group/study.

A two-way ANOVA was used to examine differences in average lesion scores between groups.

2.6. Institutional Animal Care and Use Committee (IACUC) approval

Studies presented here were pre-reviewed and approved by the University of Arizona IACUC, under protocol number 02-204.

3. Results

Parenteral immunization with HIS-rCPA partially protected birds against experimental challenge (Table 1). Differences in the percent of birds with gross lesions were statistically significant across treatment groups ($p < 0.0001$), and the percent of birds developing severe lesions (scores 3 or 4) were also significantly different across all groups ($p < 0.0095$). The differences between average lesion scores for all three groups were also statistically significant ($p < 0.0001$). The mortality rate in adjuvant only group was higher than in vaccinates, but the difference was not statistically significant (data not shown).

Birds had high average titers of anti-CPA antibodies at hatching (average titer: 120.5 ± 3.3) (Table 2), but these maternal antibodies decreased in titer during the studies, as demonstrated by titers in negative (non-vaccinated, non-challenged: 19.9 ± 2.5) and positive controls (adjuvant only, challenged: 11.7 ± 3.7) at 29 days of age. In contrast, titers in vaccinated birds were 5-fold higher (post-vaccination, pre-challenge) (183.8 ± 4.5), and 15-fold higher at necropsy (305.5 ± 5.7) compared to the adjuvant only birds.

4. Discussion

Protection against poultry NE by vaccination with CPA toxoids has been controversial. On the one hand, Lovland et al. (2004) vaccinated hens with crude “type A” or “type C” toxoids (containing CPA) and progeny were protected against subclinical NE. However, this provides little information about the role of an anti-CPA response in protection; these toxoids contained many other antigens against which the birds may have produced a protective immune response. On the other hand, birds inoculated with a CPA mutant were protected against subsequent

Table 2
Anti-CPA antibody response of birds following vaccination with recombinant CPA and challenge with *C. perfringens*

Treatment	Titers		
	Hatching ($d = 1$)	Post-vaccination ($d = 24$)	Post-challenge ($d = 29$)
rCPA vaccination	120.5 ± 3.3 ($n = 22$)	183.8 ± 4.5 ($n = 5$)	305.5 ± 5.7 ($n = 28$)
Adjuvant only, challenged		34.8 ± 1.4 ($n = 5$)	11.7 ± 3.7 ($n = 17$)
Non-vaccinated, non-challenged		17.4 ± 1.8 ($n = 5$)	19.9 ± 2.5 ($n = 33$)

End-point ELISA titers are reported as the reciprocal of the last dilution which had an OD₄₉₀ of ≥ 0.400 . Vaccinations were administered at 5 and 15 days of age.

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challenge with a virulent isolate (Thompson et al., 2006). Furthermore, birds challenged with a *cpa*-mutant NE strain, containing an 890 base pair deletion in *cpa*, developed disease at a rate and severity comparable to that in birds challenged with the parent strain (Keyburn et al., 2006). Thus, the role of CPA in pathogenesis of and immunity to NE has been called into question.

The results from this study suggest that anti-CPA immunity provides at least partial protection against experimental challenge with *C. perfringens*, and as such supports the findings of others (Kulkarni et al., 2007). They identified CPA as an immunogenic protein secreted by chicken-virulent *C. perfringens* isolates. Broilers immunized intramuscularly with histidine-tagged recombinant CPA and boosted with active toxin were significantly protected against a severe challenge. The relatively low degree of protection in our experimental trials may relate to differences in severity of challenge, and may translate into better protection against natural challenge in the field. Better responses may result from use of different adjuvants, higher doses of immunogen, or alternate routes of delivery. In this study, birds were vaccinated SQ, which typically generates a strong IgG (IgY) response. IgG (IgY) has a key role in the immune response to NE in broiler chickens (Lovland et al., 2003, 2004), and for this reason, we did not examine the IgA response of the birds. Stimulation of a strong IgA response by mucosal immunization might provide better protection.

Maternal antibodies may have interfered with the immune response to the vaccine. This study used commercial birds, as specific pathogen free (SPF) birds are not free of *C. perfringens*. Thus, colonization of hens by *C. perfringens* likely generated maternal anti-CPA antibodies that were passed into yolk, as evidenced, by high anti-CPA titers in newly hatched chicks. These maternal antibodies diminished at different rates, as shown by varying anti-CPA titers in the adjuvant only and non-vaccinated groups at the post-vaccination and post-challenge stages. Interference of maternal antibodies with vaccination might explain the minimal 5-fold increase in anti-CPA titers in vaccinates, as compared to birds in the adjuvant only group post-vaccination. Further vaccine studies with broiler chicks having low or no maternal anti-CPA titers might increase vaccine effectiveness and protection against experimental challenge.

It remains to rationalize the results of this work with those of Keyburn et al. (2006), in which CPA was suggested to be an unnecessary attribute in pathogenesis of NE. It may be that other attributes are required for establishment of infection and initiation of lesion development, and that CPA adds to the severity of disease.

In conclusion, vaccination of broiler chickens with recombinant CPA stimulates the production of anti-CPA IgG (IgY), and these anti-CPA antibodies provide partial protection against experimental challenge with *C. perfringens*. Thus, CPA appears to have a role in the pathogenesis of NE in poultry, but the extent of that role needs further investigation.

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