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Chicken immune response following *in ovo* delivery of bacterial flagellin

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ABSTRACT

In ovo immunization of chicken embryos with live vaccines is an effective strategy to protect chickens against several viral pathogens. We investigated the immune response of chicken embryos to purified recombinant protein. *In ovo* delivery of *Salmonella* flagellin to 18-day old embryonated eggs resulted in elevated pro-inflammatory chIL-6 and chIL-8 (CXCL8-CXCLi2) cytokine transcript levels in the intestine but not in the spleen at 24 h post-injection. Analysis of the chicken Toll-like receptor (TLR) repertoire in 19-day old embryos revealed gene transcripts in intestinal and spleen tissue for most chicken TLRs, including TLR5 which recognizes *Salmonella* flagellin (FliC). The *in ovo* administration of FliC did not alter TLR transcript levels, except for an increase in intestinal chTLR15 expression. Measurement of the antibody response in sera collected at day 11 and day 21 post-hatch demonstrated high titers of FliC-specific antibodies for the animals immunized at the late-embryonic stage in contrast to the mock-treated controls. The successful *in ovo* immunization with purified bacterial antigen indicates that the immune system of the chicken embryo is sufficiently mature to yield a strong humoral immune response after single exposure to purified protein. This finding strengthens the basis for the development of *in ovo* protein-based subunit vaccines.

1. Introduction

Protection of chickens against bacterial and viral pathogens is important for animal and human health. An effective and convenient protection strategy is active immunization of embryonated eggs [1–4]. During this procedure, the vaccine is injected into the amniotic sac or intramuscularly into the chicken embryo usually at 3 days prior to hatch i.e., at day 18 of embryonic development (ED18) [5]. The immunization evokes antibodies directed against the vaccine antigens, resulting in protection early after hatching. *In ovo* vaccination is commercially widely used to protect against viral infections. Most licensed vaccines consist of live attenuated viruses that can still replicate and provoke an immune response but do not cause illness [6]. More recently, non-replicating adenovirus-vector based vaccines have been developed [7,8]. *In ovo* delivery of subunit vaccines that consist of a mixture of purified antigens is still in its infancy. Successful *in ovo* immunization has been achieved with recombinant *Eimeria* protein [9–11] but immunization with a recombinant protein of *Campylobacter jejuni* failed to induce a significant immune response [12]. The reason for the variable immune response to recombinant bacterial proteins after *in ovo* delivery is unknown.

One factor that aids the generation of a potent immune response upon immunization is the use of vaccine adjuvants or other immunomodulatory agents such as cytokines. These compounds promote the immunogenicity of vaccine antigens and influence the quality of the adaptive immune response [13–15]. The repertoire of potential adjuvants for use in chickens was boosted by the discovery of functional chicken Toll-like receptors (TLR) [16,17]. Members of the TLR family of pathogen recognition receptors sense microbial ligands and translate these signals into pro-inflammatory signals that promote amongst others antigen presentation by dendritic cells, and T- and B-cell responses [18–20]. TLR agonists are beginning to be applied as vaccine adjuvants in humans [21,22], but also in the chicken [23]. The effect of TLR stimulation on the immune response seems most effective when the antigen of interest has intrinsic TLR-stimulating activity or is conjugated to an effective TLR agonist.

TLR ligands that are investigated as adjuvants in chickens include flagellin [24,25] and CpG oligodeoxynucleotides [10,26,27] .These compounds target chTLR5 and chTLR21 receptors, respectively [28–31]. A prerequisite for the use of TLR agonists

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as an adjuvant in combination with *in ovo* vaccination is the expression and function of the relevant TLR receptors at the late embryonic stage. Transcriptional profiling of immune genes during chicken embryo development indicates early but variable presence of TLR transcripts throughout the embryonic development [32–34].

In the present study we investigated the expression of TLR genes at the day of *in ovo* immunization and the effect of *in ovo* delivery of a recombinant bacterial antigen with intrinsic TLR5 stimulating activity on the generation and duration of an antigen-specific humoral immune response. We provide evidence that a single-dose injection of recombinant *Salmonella* flagellin into the amniotic sac of chicken embryos results in an intestinal cytokine response and the induction of specific IgY antibodies that can easily be detected up to 21 days post-hatch.

2. Materials and methods

2.1. Construction, expression, and purification of recombinant Salmonella His-tagged flagellin

Recombinant flagellin (FliC) of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) *was* produced as previously described [35] with some minor modifications. Briefly, the *fliC* gene of *S.* Enteritidis strain 90-13-706 was amplified by PCR as described [29], cloned with an N-terminal 6xHis-tag into the pT7.7 protein expression vector [36], and transformed into *E. coli* BL21 star (DE3). Protein expression was induced by adding 1 mM of IPTG to bacteria (OD_{550} of 0.4) grown (37 °C) in LB broth containing 100 μg/ml of ampicillin. Four hours after induction, bacteria were harvested by centrifugation and resuspended into urea solution (8 M urea in 100 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8). After 16 h of incubation (20 °C, constant rotation), the insoluble fraction were removed by centrifugation. The supernatant containing the FliC protein was mixed (1 h) with Ni-NTA beads (Qiagen). After washing of the beads with 4 × 4 ml of 8 M urea solution with pH 6.3, bound FliC protein was eluted with 4 × 0.5 ml of 8 M urea solution with pH 5.9 and, subsequently, with 4 × 0.5 ml of 8 M urea solution with pH 4.5. The FliC containing fractions were determined by 12.5% SDS-PAGE, pooled, and stored in 4 M urea solution. Protein concentrations were measured with the Pierce BCA protein assay kit.

2.2. Animal experiments

Fertilized eggs from SPF (Ross 308) broilers (Gezondheidsdienst, Deventer, the Netherlands) were kept at 38 °C and 65–75% relative humidity in a forced air egg incubator. At embryonic day 18, the eggs were candled to check their fertilization, and then divided into three groups of 15 eggs. Group 1 received 20 μg of FliC protein diluted in 100 μl of 10 mM Tris (pH 9.0)/20% glycerol/5 mM sucrose/80 mM urea (FliC group). Embryonated eggs of group 2 were injected with 100 μl of the same solution lacking FliC protein (mock group). The eggs of group 3 were kept intact and received no treatment (non-injected group). For *in ovo* delivery we followed the procedure described by Sharma [1]. Briefly, after cleaning the eggs with 0.5% hypochlorite (bleach), a small hole was made at the air cell end of the egg using an 18G sterile needle. A 22G one-inch bevel needle (Monoject) was then used to manually deliver 20 μg of flagellin (or solvent) through the air sac membrane directly into the amniotic fluid. After 24 h of incubation (to allow transport to the embryo), the embryos of five eggs from each group were aseptically removed to isolate the gut and spleen tissue. Organ samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. The remaining eggs were kept in the incubator until hatch. After hatch, the chickens were reared in a ground stable under controlled hygienic conditions for up to 21 days. Chickens were given access to water and commercial broiler diet *ad libitum* without antibiotics or coccidiostats. At day 11 post-hatch, blood samples were taken from the wing vein for antibody analysis. At day 21 post-hatch, all chickens were sacrificed by electrocution and blood was collected by exsanguination. After blood clotting and centrifugation (2000×*g*, 5 min, 4 °C), sera were collected and stored at -20 °C until analyzed. The entire experiment was repeated in the same setup with eggs from a commercial (non-SPF) flock (Lagerweij, Lunteren, the Netherlands). The *in ovo* immunization procedure did not influence the hatchability and chicken survival. All experiments were conducted in accordance with protocols approved by the Dutch experimental animal committee (DEC).

2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated from 50 (±5) mg of the collected embryonic tissue specimens. Samples were homogenized (6500×*g* for 50 s at 4 °C) in a MagNA Lyser instrument (Roche) using Lysing Matrix D tubes (MPbio) filled with 1 ml of RNA-Bee (Bio-connect USA). Total RNA was extracted using the RNA-Bee isolation kit according to the instructions of the manufacturer. The quantity and purity of the extracted RNA was measured at 260/280 nm in a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). After treatment with DNAse (1 U/μg of RNA, Fermentas), one microgram of RNA was reverse transcribed to cDNA using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Transcripts of chicken *TLR* genes and (as control) the chicken *GAPDH* gene (*chGAPDH*), were determined by PCR using the primers listed in Table 1 [37]. In all cases, RT-negative control samples were run to verify the absence of contaminating DNA. PCR amplification was performed using 1 μl of cDNA, 200 nM of each primer, 1 mM of dNTPs, and 1 Unit of Taq DNA polymerase (Fermentas) in a total reaction volume of 20 μl. The following cycle conditions were used: one initialization step at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 35 s, followed by one cycle at 72 °C (10 min). RT-PCR products were resolved by electrophoresis using 2% TBE agarose gels stained with ethidium bromide and imaged under UV illumination (Pharmacia Biotech). Results shown

Table 1
Chicken RT primers used in this study [37].

RNA target		Primer sequence (5′-3′)
chTLR1b	Forward	AGAAAAGGCTCCAGGCTACGA
	Reverse	TACGACGTTCCCCAGTTGTGT
chTLR2t1	Forward	TGAAGCTGATGTGGAAGCA
	Reverse	ACACCGTGATTTTGCCTGTGA
cTLR2t2	Forward	GATGCCAGCGCCAATAACTTTA
	Reverse	TCCATCAGTGACAGCTGCACA
chTLR3	Forward	GCACCTGTGAAAGCATTGCTT
	Reverse	AATGGAGCACTGTCCTTGCA
chTLR4	Forward	CCTGAAATGGGTCAAGGAAAAG
	Reverse	CTGTGGTTGGGTTGGGATG
chTLR5	Forward	GAAATTGGAACACAACGCGTC
	Reverse	CGGAGTATGGTCAAACGTTGC
chTLR7	Forward	GGCTGTGAATGAATGAATGGGTGA
	Reverse	GCTGAATGCTCTGGGAAAGG
chTLR15	Forward	GAAAATAAGCCCTTCGATGCCT
	Reverse	TGTTGCCAAGTAACAGGATGCC
chTLR16	Forward	TTGCTTGCACGTCTTCGACAT
	Reverse	TTAGGAAGACCGTGTCCAGGTG
chTLR21	Forward	GCAGCTAGCCGCTCCTTTT
	Reverse	CCTTCTTCTTCCTCCTCCTCTCC
chGAPDH	Forward	TGCTGTGCTGGCCATCTTT
	Reverse	GCTTTGGACGTGCTCCAAA

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