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Reduction of avian influenza virus shedding by administration of Toll-like receptor ligands to chickens

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ABSTRACT

Avian influenza viruses (AIV) are of concern to the poultry industry. Outbreaks of AIV highlight the urgent need for effective control measures. Prophylactic strategies should be explored that rapidly elicit immunity against the virus. Toll-like receptors (TLRs) are innate immune molecules that can induce antiviral responses, therefore the application of TLR ligands as prophylactic agents in chickens is gaining more attention. We hypothesized that treatment of chickens with TLR ligands reduces the shedding of AIV from infected birds. In addition, the effects of TLR ligand dose and route of administration on the efficiency of TLR ligands to reduce AIV shedding were examined. Chickens were treated with TLR2, 4, 7 and 21 ligands using different doses and routes of administration, 18 h before AIV infection. Moreover, the expression of several candidate genes, such as type I interferons, PKR, OAS, viperin and IFITM3 was quantified at 3, 8 and 18 h post-treatment with TLR ligands. The results revealed that route of administration and dosage affect the efficacy of TLR ligands to reduce virus shedding. Furthermore, varying effects were observed when different ligands were applied. Our results demonstrate that all TLR ligand treatments reduced AIV shedding, with the CpG-ODN 1826 being the most efficacious to reduce oral virus shedding, whereas LPS from *Escherichia coli* 026:B6 resulted in the largest reduction in cloacal virus shedding. Moreover, TLR ligands induced the expression of genes involved in antiviral responses such as type I interferons and interferon-stimulated genes in chicken trachea and cecal tonsils. These results raise the possibility of treatment of chickens with TLR ligands as anti-viral agents.

1. Introduction

Avian influenza viruses (AIVs) are a concern for animal and human health. Several strategies are currently employed for control of AIV, including biosecurity measures and vaccination. Other approaches that rely on induction of host innate antiviral responses have also been evaluated experimentally [1]. These approaches can complement current control strategies and provide quick responses especially in outbreak situations. In this regard, ligands of pattern recognition receptors (PRRs) may be employed for induction of innate responses. PRRs recognize pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are the best characterized members of the PRR family [2]. Ten TLRs are present in chickens [3]. The interaction of TLRs with PAMPs leads to activation of intracellular signaling pathways and expression of type I interferons (IFNs) and pro-inflammatory cytokines [4]. Subsequently, the binding of IFNs to their receptors results in the transcription of interferon-stimulated genes (ISGs) [5]. ISGs encode proteins that have antiviral properties which can interfere with viruses at different stages of the virus replication cycle, such as blocking entry of virus into host cells, virus assembly inhibition, abolishing of translation processes, blocking of virus budding, and release of newly synthesized virus. The direct antiviral function of some of these proteins, such as RNA-activated protein kinase (PKR), IFN-induced transmembrane proteins (IFITM) and viperin against AIV, has been defined [5–7].

The immunostimulatory properties of TLR ligands have been studied in mammalian species and chickens [3,8]. TLR ligands have been used in mammals as therapeutic or prophylactic agents against several infectious diseases [9–11]. TLR ligands have been employed in chickens as adjuvants to increase vaccine efficacy [12–15]. Previously, we have shown that TLR2, 3, 4, 5 and 21 ligands, as adjuvants in an AIV vaccine, can increase antibody- and cell-mediated immune responses, and significantly reduce shedding

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of AIV in vaccinated chickens [14,15]. Specifically, CpG is a more potent adjuvant in chickens to induce immune responses against AIV compared to other TLR ligands [15]. The protective ability of TLR ligands is not limited to their application as adjuvants. TLR ligands can be employed as stand-alone antibacterial or antiviral agents. For example, TLR2, 4 and 21 ligands significantly reduce replication of AIV in chicken macrophages [16]. TLR2 and 9 ligands protect mice against lethal influenza virus infection [11], and TLR2, 3, 4, 21 ligands protect against bacterial and viral infections [3]. We have also demonstrated that intramuscular administration of TLR3, 4 and 21 ligands (polyI:C, lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) 0111:B4 and class B CpG, respectively) significantly reduce cloacal and oral AIV shedding [17].

Several factors, such as route of administration or dosage significantly affect the pharmacokinetics and efficacy of TLR ligands [18]. In the present study, the effect of dose and route of TLR2, 4, 7 and 21 ligand administration on the potential to induce antiviral responses and subsequently to reduce AIV shedding in chickens was investigated. In addition, the efficacy of different ligands within a family, including different classes of CpG or different sources of LPS, was compared.

2. Materials and methods

2.1. Chickens

One-day-old specific pathogen-free (SPF) chickens were housed in the animal isolation facility of the Ontario Veterinary College, University of Guelph. The research was approved by the University of Guelph Animal Care Committee, and adhered to the guidelines of the Canadian Council for Animal Care.

2.2. Avian influenza virus

A/Duck/Czech/56 (H4N6), a low pathogenic avian influenza virus (LPAIV), was used. The virus was propagated in 11-day-old embryonated chicken eggs by inoculation into the allantoic cavity [19]. Virus titer in allantoic fluid was determined at 72 h post-inoculation and expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml [20].

Table 1
TLR ligands and their corresponding doses.

TLR ligand	TLR	High dose (μg/chicken)	Low dose (μg/chicken)
Pam3CSK4	TLR2/1	100	20
LPS (*E. coli* 0111:B4)	TLR4	500	100
LPS (*E. coli* 026:B6)	TLR4	500	100
R848	TLR7	100	20
CpG ODN 2216	TLR21	50	10
CpG ODN 2007	TLR21	50	10
CpG ODN 1826	TLR21	50	10
CpG ODN 2395	TLR21	50	10
Non-CpG ODN	50		

These doses were selected based on previous studies and manufacturer's protocols [17,23,36].

2.3. TLR ligands

Pam3CSK4 and R848 were purchased from Invivogen (San Diego, CA, USA). Class A CpG 2216, class B CpG 2007, class B CpG 1826, class C CpG 2395, non-CpG ODN, LPS from *E. coli* O111:B4 and LPS from *E. coli* 026:B6 were purchased from Sigma-Aldrich (Oakville, ON, CA). These ligands were selected as they have previously been shown to stimulate chicken TLRs [3,17]. We did not select poly I:C, a ligand for TLR3, for the present study because it is less stable. Also, given the cost of this compound, we reasoned that it may not be directly applied to poultry industry in the near future.

2.4. Experimental design

In the first trial, to assess the effect of TLR ligands on shedding of H4N6 AIV, 360 fourteen-day-old chickens were randomly divided into 36 groups ($n = 10$/group). Chickens received two different doses of the TLR2, 4, 7 and 21 ligands Pam3CSK4, LPS, R848 and CpG, respectively, while control groups received non-CpG or PBS (Table 1). On day 14 of age, ligands were administered either intramuscularly or intranasally. TLR ligand doses were selected based on previous experiments in chickens [3,17]. Eighteen hours post-treatment, chickens were infected with 1×10^6 $TCID_{50}$ H4N6 AIV oculo-nasally in 100 μl. Oropharyngeal and cloacal swabs were collected from each group at 4 and 7 days post-infection (dpi), respectively. Virus titers in the swabs were determined by $TCID_{50}$ [20].

Table 2
Primer sequences used for real-time PCR.

Gene name	Primer sequence	Annealing temp. (°C)	Reference
β-actin	F: 5′-CAACACAGTGCTGTCTGGTGGTA-3′ R: 5′-ATCGTACTCCTGCTTGCTGATCC-3′	60	[41]
Interferon-α	F: 5′-ATCCTGCTGCTCACGCTCCTTCT-3′ R: 5′-GGTGTTGCTGGTGTCCAGGATG-3′	64	[41]
Interferon-β	F: 5′-GCCTCCAGCTCCTTCAGAATACG-3′ R: 5′-CTGGATCTGGTTGAGGAGGCTGT-3′	64	[43]
PKR	F: 5′-TGGTACAGGCGTTGGTAAGAG-3′ R: 5′-GAGCACATCCGCAGGTAGAG-3′	60	This paper
IFITM3	F: 5′-CACACCAGCATCAACATGCC-3′ R: 5′-CCTACGAAGTCCTTGGCGAT-3′	60	This paper
OAS	F:5′-AGAACTGCAGAAGAACTTTGTC-3′ R:5′-GCTTCAACATCTCCTTGTACC-3′	60	[43]
Viperin	F: 5′-GGAGGCGGGAATGGAGAAAA-3′ R: 5′-CAGCTGGCCTACAAATTCGC-3′	60	This paper
MDA5	F: 5′-GCAAAACCAGCACTGAATGGG-3′ R: 5′-CGTAAATGCTGTTCCACTAACGG-3′	60	This paper
TLR3	F: 5′-TCAGTACATTTGTAACACCCCGCC-3′ R: 5′-GGCGTCATAATCAAACACTCC-3′	64	[30]
TLR7	F: 5′-TTCTGGCCACAGATGTGACC-3′ R: 5′-CCTTCAACTTGGCAGTGCAG-3′	64	[30]

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