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Synthetic double-stranded RNA oligonucleotides are immunostimulatory for chicken spleen cells

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ABSTRACT

Toll-like receptors (TLRs) are an integral part of the innate immune system that recognize microbe-derived molecular patterns and initiate innate and adaptive defenses against invading pathogens. TLR3 and TLR7 are involved in sensing virus-associated single-stranded and double-stranded RNA (dsRNA) molecules in cellular endosomes to activate the type I interferon pathway in mammals. Although certain synthetic dsRNA molecules have been identified to show immunostimulation in mammalian cells, very little is known about the ability of these sequences to stimulate avian cells. The current study investigated immunostimulatory properties of four synthetic oligonucleotide sequences using chicken splenocytes. Expression of TLR3 and 7, type I interferons and several other cytokines as well as TLR signaling pathway-related genes at different time points post-stimulation was quantified by real-time PCR. A dose-dependent increase in expression of TLR3 was observed in splenocytes treated with poly-UGUGU (poly-UG) and β-galactosidase dsRNA molecules. TLR3 and TLR7 gene expression was significantly up-regulated upon stimulation with all four dsRNA molecules. Furthermore, in a time course study, a significant increase was noted in the expression of TLR3, TLR7, interferon (IFN)-α, IFN-β, interleukin (IL)-1β, IL-6 as well as 2′,5′-OAS in splenocytes treated with poly-UG. In conclusion, the present study demonstrated the immunostimulatory properties of dsRNA oligonucleotides, especially those that contain a poly-UG motif, in chickens.

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1. Introduction

The TLR family of pathogen recognition receptors is a diverse and important part of the innate immune system in vertebrates. TLRs recognize PAMPs derived from a variety of microorganisms, including bacteria and viruses. Chicken TLRs orthologous to human TLRs 1, 2, 3, 4, 5, and 7 have been identified (Brownlie et al., 2009; Iqbal et al., 2005; Yilmaz et al., 2005). Chickens also have certain TLRs that do not have significant homology with human TLR sequences, but recognize similar PAMPs. For example, chicken TLR21 has been demonstrated to play a role similar to that of human TLR9 in recognizing CpG DNA motifs from viruses or bacteria (Brownlie et al., 2009). TLRs contain multiple leucine-rich repeats in their ligand-binding ectodomain and recognize various PAMPs, ranging from extracellular structures (e.g., lipopolysaccharide) to nucleic acids, such as dsRNA (Jin and Lee, 2008). Most TLRs trigger a signaling pathway, which involves MyD88. However, signaling triggered by TLR3, is dependent on the TRIF co-adaptor molecule (Brikos and O’Neill, 2008).

TLR3 is primarily localized in endosomes and recognizes dsRNA molecules of at least 40–50 bp in length (Liu et al., 2008). However, recent findings have also suggested an additional dsRNA binding site on human TLR3 that can bind RNA duplexes as short as 21 bp (Pirher et al., 2008). Further, Kleinman et al. (2008) reported that shorter siRNA molecules can bind cell-surface TLR3 in mice. Poly(I:C), a dsRNA analogue, is a well-characterized TLR3 agonist and a potent inducer of type I interferons (IFNs) in many cell-types including myeloid dendritic cells, intestinal epithelial cells and fibroblasts (Matsumoto et al., 2004). TLR7, another sensing receptor of viral nucleic acids, can be activated by sequence-specific ssRNA as well as dsRNA duplexes of 19–21 bp in length (Heil et al., 2004). Various ssRNA and siRNA-sized dsRNA molecules have been used to activate mammalian TLR7 (Judge et al., 2005). In chickens, TLR7 is suggested to be present mainly in B cells and T cells (Iqbal et al., 2005) and ligation of both TLR3 and 7 with their ligands results in type I IFN production (Judge et al., 2005; Karpala et al., 2008;
Phibin et al., 2005). Type I IFNs, which include IFN-α and IFN-β, are released in large quantities by the immune system cells, such as plasmacytoid dendritic cells, lymphocytes, macrophages, natural killer cells, and in lesser quantities by most other cell types, such as fibroblasts. Type I IFNs are usually expressed by host cells in response to viral infections and they induce a generalized antiviral state in infected cells as well as in neighboring cells (Kindt et al., 2006).

Recent studies have demonstrated that induction of antiviral cytokine secretion in human and murine immune system cells is dependent on the sequence and dose of the TLR ligands, namely ssRNA and dsRNA (Diebold et al., 2006; Hornung et al., 2005; Judge et al., 2005; Robbins et al., 2008; Sioud, 2005). However, very little work has been done to investigate this phenomenon in chickens. Using four immunostimulatory siRNA-like dsRNA molecules reported in previous murine studies, we tested the sequence- and dose-dependency of type I IFN response to stimulation by these 19–21 bp dsRNA oligonucleotide molecules in chicken splenocytes. We then further investigated temporal effects of stimulation by one of the candidate dsRNA molecules on spleen cells.

2. Materials and methods

2.1. Experimental animals

Five- to six-week-old SPF chickens, homozygous for the B19 MHC haplotype, were provided by Cornell University, Ithaca, NY. All chicks were kept in the Isolation Unit of Ontario Veterinary College (University of Guelph, Guelph, Ontario, Canada).

2.2. dsRNA sequences and motifs

RNA duplexes were obtained from Integrated DNA Technologies Inc. (Coralville, IA) in 2 nmol quantities. Each of the oligonucleotide molecules was resuspended in TE buffer (pH 8.0) to a stock concentration of 100 μM and stored at –20°C. Poly(I:C) (sodium salt, γ-irradiated) was obtained from Sigma–Aldrich, St. Louis, MO. Sequences of siRNA-based RNA duplexes were selected from previous research conducted on human blood mononuclear cells. The SARS coronavirus-targeting dsRNA molecule (siSC2) and the β-galactosidase-targeting dsRNA molecule (β-gal) were found to be immunostimulatory in vitro studies (Judge et al., 2005; Robbins et al., 2008). β-gal dsRNA was suggested to contain an immunostimulatory motif, “UGUGU” (Judge et al., 2005). To take this further, we designed a 21-mer dsRNA molecule comprised completely of UG repeats on the sense strand (Table 1). The dsRNA molecule targeting enhanced green fluorescent protein (EGFP) has been used as a negative control molecule for immune reactivity (Robbins et al., 2008).

2.3. Primary cell culture

Spleens were harvested from 5- to 6-week-old B19 chickens and spleen mononuclear (spleocytes) cells were isolated on Histopaque® 1077 (Sigma–Aldrich, St. Louis, MO). Splenocytes were cultured in RPMI 1640 medium supplemented with 10% FBS, 0.5% gentamycin, and 1% penicillin/streptomycin during the experiment.

2.4. Experimental design

Two sets of experiments were performed. The first set was comprised of cells stimulated with dsRNA molecules for 4 h, where the cells were collected for RNA extraction at two time points (2 and 4 h post-stimulation). The splenocytes received three concentrations of each of the dsRNA molecules, namely 25, 50, or 100 nM. Control cells were mock-treated with the transfection reagent, PEI. In the second experiment, cells were stimulated with either 50 nM poly-UG or 50 μg/mL poly(I:C). Cells were collected for RNA extraction at 1, 3, 6, 12, and 18 h post-stimulation. Control cells were mock-treated with PEI. Both of the experiments were performed in triplicate.

2.5. Transfection of splenocytes

For the transfection experiments, resuspended RNA duplexes (with a final concentration of either 25, 50, or 100 nM) were added to 75 μL of RPMI and incubated at room temperature for 15 min before 30 μg of PEI was added and incubated for another 15 min. Complete RPMI was then added to make a total of 1.2 mL of transfection medium. After transfection, cells were plated at 1.0 × 10^6 cells in a volume of 500 μL in 24-well plates and incubated at 41°C, 5% CO₂. One group was treated with poly(I:C) suspended in physiological saline to a final concentration of 50 ng/μL.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA). RNA quality and quantity were estimated spectrophotometrically. Five hundred ng of RNA were reverse-transcribed into cDNA using the SuperScript® First-Strand kit.
2.7. Real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, Germany) on 384-well and 96-well plates in a 20 μL reaction volume as outlined by Parvizi et al. (2009). Primers for β-actin, TLR3, TLR7, TRIF, 2′-5′-oligo-adenylate synthetase (OAS), interleukin (IL)-1, IL-6, IL-12p40, IFN-α, IFN-β, and IFN-γ were synthesized by Sigma–Aldrich Canada, Oakville, ON and their sequences are given in Table 2.

2.8. Data analysis

Relative expression was calculated using the Pfaffl’s formula (Pfaffl, 2001) as described previously (Parvizi et al., 2009). Briefly, relative expression of all genes was calculated based on the expression of the house keeping gene, β-actin. Absolute quantification of β-actin expression was estimated using the LightCycler® 480 software (Roche Diagnostics GmbH, Mannheim, Germany). Absolute expression of all genes tested was then normalized to the expression of β-actin.

Triplicate data for relative expression of genes to β-actin was analyzed with the Student’s t-test. To test for dose- and time-dependency, experimental data analyzed with ANOVA. Comparisons were considered significant at \( p \leq 0.05 \).

3. Results

3.1. Effects of short-term stimulation of cells with dsRNA oligonucleotides

A significant increase in the transcription of TLR3 and TLR7 genes at 2 h post-stimulation was observed in splenocytes stimulated with 50 nM of poly-UCUGU (poly-UG) dsRNA (Fig. 1A and B). Although statistically not significant (\( p = 0.06 \)), a trend of increase in IFN-α gene transcription was evident at 2 h post-stimulation in cells treated with 50 nM of poly-UG dsRNA (Fig. 2A). In addition, cells treated with siSC2 dsRNA at 25 and 50 nM were also found to up-regulate TLR3 transcription significantly at 2 h post-stimulation. However, a significant increase in TLR7 gene expression was observed in cells treated with 25 nM of siSC2 dsRNA at 2 h post-stimulation and cells treated at 50 and 100 nM concentrations showed significant up-regulation of TLR7 at 4 h post-stimulation (Fig. 1B). There was no significant increase in IFN-α transcription in cells stimulated with siSC2 dsRNA at any given time points (Fig. 2A). β-gal treatment of splenocytes at a concentration of 25 nM was shown to up-regulate both TLR3 and 7 expression at 4 h post-
A dose-dependent up-regulation of TLR3 and 7 genes expression in cells stimulated with poly-UG and β-gal dsRNA was observed, as analyzed by ANOVA. Poly-UG dsRNA-treated splenocytes showed significant dose-dependent up-regulation in expression of TLR3 and TLR7 genes at 2 h post-stimulation, whereas cells treated with β-gal dsRNA displayed dose-dependent transcription of the TLR7 gene at 4 h post-stimulation (Fig. 1A and B). However, no significant dose-dependent increase in IFN-α gene transcription was observed in response to stimulation by any of the dsRNA oligonucleotides. These findings suggested that up-regulation of TLR3 and 7 expression by dsRNA molecules is not only sequence-dependent but is also dose-dependent.

Further investigation to address changes in the TLR-associated co-adaptor gene expression in splenocytes stimulated with dsRNA molecules showed significant up-regulation of TRIF gene expression in cells stimulated with poly-UG dsRNA at 4 h post-stimulation (Fig. 1C). However, no significant changes in MyD88 gene expression levels were observed in cells stimulated with any of the dsRNA molecules (data not shown). Similarly, no significant differences were found in the expression of either IL-6 or IL-12 p40 in any of the treated groups (data not shown).

### 3.2. Effects of poly-UGUGU dsRNA on spleen cells

Based on the above findings that showed increased expression of TLR3 and 7 genes as well as IFN-α genes at early time points in splenocytes stimulated with poly-UG dsRNA (50 nM), further experiments investigated the temporal effects of poly-UG-mediated stimulation in these cells. The time points for collecting splenocytes included 1, 3, 6, 12 and 18 h post-stimulation. A summary of findings from this investigation is provided in Table 3. Significant up-regulation of IFN-α transcription in stimulated cells was observed at 1, 12 and 18 h post-stimulation compared to unstimulated controls (Fig. 3A). Although statistically not significant, a trend of increase in the IFN-α gene transcription was also observed at 3 and 6 h post-stimulation. Poly-UG dsRNA stimulation also resulted in significant increase in the levels of 2′,5′-OAS transcripts at 12 and 18 h post-stimulation (Fig. 3C). The levels of IFN-α transcripts in cells treated with poly-UG at 12 and 18 h post-stimulation were significantly higher compared to poly(I:C) treated cells at these time points (Fig. 3A). Further, poly-UG dsRNA treated cells significantly up-regulated TLR3 and 7 transcripts at 18 h post-stimulation and in addition, TLR7 gene expression was also found to be significantly up-regulated as early as 1 h post-stimulation (Fig. 4A and B). A trend of increase in TLR3 and 7 transcript levels was evident at 3, 6 and 12 h post-stimulation suggesting a time-dependent increase in transcription of these genes in response to poly-UG dsRNA stimulation of splenocytes.

Poly-UG dsRNA stimulation of splenocytes was further shown to significantly up-regulate expression of pro-inflammatory cytokine

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**Table 3**

Summary of cytokine gene transcription in chicken splenocytes stimulated by the dsRNA molecules poly-UG and poly(I:C) after stimulation for up to 18 h.

| Treatment  | Hours | IFN-α | IFN-β | IFN-γ | TLR3 | TLR7 | IL-6 | IL-1β | OAS |
|------------|-------|-------|-------|-------|------|------|------|-------|-----|
| Poly-UG 50 nM | 1     | *    | 0     | 0     | 0    | 0    | 0    | 0     | 0   |
|            | 3     | 0    | 0     | 0     | 0    | 0    | 0    | 0     | 0   |
|            | 6     | 0    | 0     | 0     | 0    | 0    | 0    | 0     | 0   |
|            | 12    | *    | *     | 0     | 0    | 0    | 0    | *     | 0   |
|            | 18    | *    | *     | *     | *    | *    | *    | *     | *   |
| Poly(I:C) 50 ng/μL | 1     | *    | 0     | *     | *    | *    | *    | *     | *   |
|            | 3     | *    | *     | *     | *    | *    | *    | *     | *   |
|            | 6     | *    | *     | *     | *    | *    | *    | *     | *   |
|            | 12    | *    | *     | *     | *    | *    | *    | *     | *   |
|            | 18    | 0    | 0     | 0     | 0    | 0    | 0    | 0     | 0   |

0, no significant change; *, significant increase; –, significant decrease. Statistical significance at each of the time-points was evaluated by comparing the gene expression in the treatment group to that of vehicle (PEI-) controls.
Fig. 3. Relative expression of (A) IFN-α, (B) IFN-β, and (C) OAS genes was measured 1, 3, 6, 12, and 18 h post-stimulation with poly-UGUGU dsRNA and poly(I:C) (1 and 3 h not shown) (A–E). Cells were stimulated with either 50 nM poly-UG, 50 ng/μL poly(I:C), or sham treated with PEI. Results are shown as transcription of the target gene relative to housekeeping gene, β-actin. Inset figures: only poly-UG and PEI treated cells are shown. Statistical significance was determined between treated groups and the sham-treated group (*p ≤ 0.05).

4. Discussion

The present study demonstrated immunostimulatory properties of some dsRNA molecules in chicken splenocytes. The choice of dsRNA sequences was based on the previous studies that reported immunostimulation by some of these oligonucleotides in mammalian cells (Diebold et al., 2006; Hornung et al., 2005; Judge et al., 2005; Robbins et al., 2008; Sioud, 2005). In chickens, Karpala and colleagues (2008) recently showed that treatment of splenocytes with poly(I:C) resulted in induction of type I IFNs and this effect was found to be TLR3-dependent. However, the effects of synthetic dsRNA molecules other than poly(I:C) in chicken splenocytes were not investigated. The present study showed that all four synthetic dsRNA molecules used in this study induced significant up-regulation of TLR3 and 7 transcripts at one of the early time points (2 or 4 h) post-stimulation (Fig. 2A and B). However, only poly-UG dsRNA (50 nM) was found to significantly up-regulate TLR3 at both 2 and 4 h in addition to increased TLR7 and IFN-α transcripts at 2 h post-stimulation. This observation indicated that poly-UG dsRNA oligonucleotide may have enhanced immunostimulatory effects compared to the other dsRNA oligonucleotides that were evaluated in this study. Further investigation of the immunostimulatory effects of poly-UG dsRNA revealed a significant increase in cellular expression of IFN-α, IFN-β, TLR3 and TLR7 genes at 12 and 18 h post-stimulation (Figs. 1 and 2). In the case of TLR3 and TLR7, TLR7 gene expression was also found significantly up-regulated as early as 1 h post-stimulation (Fig. 4A and B), whereas the expression of both TLR3 and 7 showed a trend (p > 0.05) towards up-regulation at 3, 6 and 12 h post-stimulation. Based on the above observation of an increased expression of TLR3 and TLR7 genes, it
may be concluded that the poly-UG-induced up-regulation of IFN-α genes is mediated by TLR3- or TLR7. In this regard, IFN-α produced by splenocytes may further up-regulate TLR3 or TLR7 by acting in an autocrine or paracrine manner (Karpala et al., 2008; Miettinen et al., 2001; Sirén et al., 2005). Induction of type I IFN production was also supported by the corresponding transcriptional elevation of the IFN-inducible gene, 2′,5′-OAS in the present study. In addition, transcriptional up-regulation of type I IFN along with other pro-inflammatory cytokine genes, IL-6 and IL-1β, was observed at 12 or 18 h post-stimulation, suggesting a delayed inflammatory response by the splenocytes.

Sequence-dependent immunostimulation seems to be an important phenomenon in virus-associated PAMP recognition by TLRs. For example, two recent studies that screened various dsRNA molecules for immunostimulation in human PBMCs, which included four of the sequences used in the present study, found that siSC2 dsRNA and β-gal dsRNA were potent inducers of type I IFN production compared to other molecules (Judge et al., 2005; Robbins et al., 2008). In contrast, these same siSC2 and β-gal dsRNAs did not show appreciable immunostimulation in the present study compared to poly-UG dsRNA, indicating that the RNA-sensing specificities of TLR3/7 may vary across species. This observation can further be supported by the findings of Philbin et al. (2005) who did not observe immunostimulation in chicken splenocytes with two ssRNA molecules that had shown type I IFN production in human peripheral blood mononuclear cells (Heil et al., 2004). This disparity in stimulatory effects of RNA sequences between mammalian and avian species may have resulted from the differences in their evolutionary development of the components of innate immune system. Therefore, it can be speculated that recognition of ligands by TLRs is governed by certain subtle species-specific differences; thus, chickens may respond to a different repertoire of immunostimulatory RNA sequences.

The findings presented here demonstrated that type I IFN response in chicken splenocytes can be induced by poly-UG dsRNA, which is 19–21 bp in length, although the response was relatively less rapid and of lesser magnitude than the response observed with poly(I:C) stimulation. This may be due to differences in the nature and sequences of RNA molecules since human PBMCs are shown to exhibit different degrees of immunostimulation when treated with sense, anti-sense, or duplex RNA molecules (Judge et al., 2005). Importantly, poly-UG dsRNA stimulation of splenocytes had more prolonged immunostimulatory effects compared to poly(I:C).

An important observation about IFN-γ gene expression in this study was a temporal expression profile in cells treated with poly(I:C). To our knowledge, this temporal expression in response to poly(I:C) stimulation has not been demonstrated in the avian system, though it has been examined in human NK cells (Duluc et al., 2009). Transcription of IFN-γ in mammals has been shown to be mediated by IFN-β in an autocrine fashion (Duluc et al., 2009). This mechanism may also exist in the chicken, because in the present study, a significant increase in IFN-β transcription was observed up to 3 and 6 h post-stimulation with a corresponding significant increase in IFN-γ. However, poly-UG dsRNA stimulation of chicken splenocytes was not associated with an increase in IFN-γ transcription in the present study, suggesting that IFN-γ expression in response to stimulation by dsRNA molecules may be questionable and may depend on the length and sequences of dsRNA oligonucleotides.

In conclusion, the findings from this study demonstrated immunostimulatory properties of certain dsRNA molecules and in particular, poly-UG dsRNA which induced activation of splenocytes through transcription of both anti-viral and pro-inflammatory cytokines. Further studies are required to investigate in vivo effects of poly-UG dsRNA to determine its usefulness as a possible immunostimulatory molecule for use in chicken vaccines.

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