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Original article

The effects of subcutaneous and intraocular administration of class B ODN CpG in chicken on the expression of TLR21, IFN-γ and IL-1β

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Abstract

The synthetic unmethylated oligodeoxynucleotides with CpG motifs (CpG ODN) were shown to activate Toll-like receptor 21 (TLR21) and stimulate the innate and adaptive immune system. In this study we tested the expression of TLR21, interferon (IFN)-γ and interleukin (IL)-1β mRNA in the blood after subcutaneous and intraocular application of the class B CpG ODN in chicken. The relative expression of mRNA of TLR21, IFN-γ and IL-1β were quantified at 3, 6, 12, 24 and 72 h post-stimulation. The study revealed that IFN-γ mRNA expression was significantly upregulated 12 h after subcutaneous stimulation with a high and low dose of ODN CpG, whereas the IL-1β mRNA expression levels were significantly upregulated 3 and 72 h after subcutaneous administration. After intraocular administration, the IL-1β mRNA levels were the highest at 24 h post-application, albeit not specifically. This data indicates that class B CpG ODN has the ability to induce TLR21 response in blood when administered parenterally in chicken. In contrast, intraocular administration of CpG ODN was not able to produce a significant increase in cytokine mRNA expression in blood. The data suggest that additional stimulus, e.g. the antigen, may be needed on the site of mucosal administration to activate systemic immune response.

Key words: ODN CpG, TLR, IFN-γ, IL-1β, chicken

Introduction

Pattern recognition receptors (PRRs), such as Toll-like receptors are the central component of the innate immune system and play a crucial role in the first line host defence against pathogens. Bacterial and viral DNA has unmethylated deoxycytidyl--deoxyguanosine dinucleotides (CpG) motifs that are recognized by TLR9 in mice and humans (Takeda et al. 2003), and by TLR21 in chickens (Brownlie et al. 2009, Keestra et al. 2010). Stimulation of TLR21 by ODN CpG induces NF-kappaB activation and therefore promotes transcription of proinflamatory cytokines (Brownlie et al. 2009).

The synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) are capable of evoking a range of immunostimulatory effects in vertebrates and have potential to be used as therapeutic

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agents. There are three major classes of CpG ODN that are grouped according to their flanking sequence motifs, classes A, B and C (Vollmer et al. 2004). Different classes of CpG ODN have been attributed to stimulate various response pathways, the class B CpG ODNs strongly stimulate B cells, thereby enhancing proliferation and antigen presentation, in addition to promoting the production of cytokines such as IFN-γ (Krug et al. 2001).

In chickens the ODN CpG can act as immunostimulants leading to increased protection against different pathogens (Vleugels et al. 2002, Linghua et al. 2007, Taghavi et al. 2008, 2009, Mallick et al. 2012). For instance, it was demonstrated that 64CpG-plasmid co-administered with the H5N2 avian influenza inactivated vaccine, increase hemagglutination inhibition (HI) titers, proliferation of peripheral blood mononuclear cell IFN-α, IFNγ mRNA gene expression and increases survival rate after challenge with a highly virulent H5N1 strain (Hung et al. 2011). It was also shown that ODN CpG can adjuvant the chicken's immune response not only when given parenterally but also when given orally or intranasally (Ameiss et al. 2006, Fu et al. 2013). Those attributes make class B CpG ODN potential immune adjuvant mediated by TLR21 pathway in avian species. However, little is known about the role of class B CpG ODN in triggering proinflamatory gene expression when given by intraocular route.

In the eye, the lymphoid associated tissue makes the first line defence against pathogens. The Harderian gland (HG) located behind the orbit of the eye plays a role in adaptive immune response, producing numerous immunoglobins and probably presenting antigens (del Cacho et al. 1992, Śmiałek et al. 2011, Gurjar et al. 2013). Since the intraocular route is widely used in vaccination of chicken with live-attenuated viruses and bacteria (Purswell et al. 2010, Gurjar et al. 2013), it is important to know if this way of administration of class B CpG ODN can elicit the proinflamatory response.

In this study we tested the hypothesis that intraocular administration of the TLR agonists, the class B CpG ODN, is able to induce the immune response in chickens, by measuring the gene expression of TLR21, IFN-γ and IL-1β in peripheral blood.

Materials and Methods

Animals

A total of 280 of 1-day-of-age healthy Hubbard Flex breeder male chicks were obtained from a commercial hatchery and were raised in cages. The chicks were not vaccinated. Lighting and ventilation were identical for all the chickens. All the birds were kept in laboratory conditions. Water and commercial feed were available *ad libitum*. The experiment was conducted with the consent of the Local Ethics Commission for Animal Experiments (No. 42/2009 and No. 77/2009).

Ligands

The synthetic class B CpG ODN (5'-TCGTCG TTGTCGTTTTGTCGTT-3') and non-CpG ODN (5'-TGCTGCTTGTGCTTTTGTGCTT-3') were purchased from TIB Molbiol, Syntheselabor GmbH (Berlin, Germany), both with a phosphorothioate backbone. Both ODNs were resuspended in sterile phosphate-buffered saline (PBS, pH 7.4) and diluted to their working concentrations in PBS.

Experimental design

In experiment 1, 140 1-day-of-age chicks were divided into four groups (ODN50, ODN25, non-ODN, PBS). The group ODN50 was injected subcutaneously with 50 µg/chick of class B CpG ODN, the group ODN25 was injected subcutaneously with 25 µg/chick of class B CpG ODN, the group non-ODN was injected with 50 µg/chicks of no-CpG ODN, the group PBS was injected with 50 µg/chick of PBS. All formulations were delivered in a total volume of 0.5 ml.

In experiment 2, 140 1-day-of-age chicks were divided into four groups (ODN50, ODN25, non-ODN, PBS) and class B CpG ODN, non-ODN or PBS were administrated (as in experiment 1) in 0.2 ml volume by intraocular route. At 3, 6, 12, 24 and 72 h post-injection randomly selected seven birds from each group were euthanized and the peripheral blood was collected for RNA extraction.

Quantitative real-time reverse transcription PCR (qRT-PCR) analysis of the expression of cytokine mRNA

The total RNA was extracted using Total RNA Mini Plus (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. Total RNA samples (1 mg each reaction) were reverse transcribed to cDNA by oligo (dT)18 and random hexamer primers using Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Massachusetts, USA). The mRNA expression levels

Table 1. The list of primers used in the study. Acc. no. — accession number.

cDNA	Acc. no.	primers F (forward), R (reverse)
TLR21	AJ720600.1	F: 5-5' TGCTGGACCTGTCTCACAAC-3' R: 5' CGTGCTGAGGGGGTTAGTT-3'
IFN-γ	Y07922	F: 5'-CCAAGAAGATGACTTGCCAGA-3' R: 5'-ACCTTCTTCACGCCATCAGG-3'
IL-1β	AJ245728	F: 5'-GGCATCAAGGGCTACAAGC-3' R: 5'-GTTGGAGCGGGCAGTCAG-3'
β-actin	X00182	F: 5'-CAACACAGTGCTGTCTGGTGGTA-3' R: 5'-ATCGTACTCCTGCTTGCTGATCC-3'

of chicken IFN-γ, IL-1β, TLR21 and a housekeeping gene, β-actin, were determined by iQ5 real-time PCR (Biorad, Hertfordshire, UK). Primers (Table 1) were obtained from Genomed (Warszawa, Poland). The amplification efficiency was verified for each gene using 2-fold serial dilutions of cDNA. Analysis of qRT-PCR was performed for each sample in duplicate in a total volume of 20 µl, consisting of 10 µl of KAPA SYBR® FAST Bio-Rad iCycler 2X qPCR Master Mix, 0.3 µl of 10 mM each of forward and reverse primers, 2 µl of target cDNA (25 ng RNA), 7.4 µl RNase/DNase-free water. All reaction plates were run under identical cycle conditions, 95°C for 3 min, and 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 min. The fluorescence threshold was set at 0.2 and the resulting cycle threshold (Ct) values, normalized to the reference gene, were used for analysis.

Data analysis

The Ct value was derived for each PCR reaction, the average Ct value for each duplicate reaction was considered for statistical analysis. The fold changes in gene expression in CpG-ODN or non-CpG ODN compared to PBS-treated control group were assessed by the formula previously described (Pfaffl 2001). Data were analyzed using Relative Expression Software Tool-Multiple condition solver (REST-MCS) beta software v. 2. The REST-MCS beta allows analysis by above formula and test the significance between groups by pairwise fixed relocation randomization test (Pfaffl et al. 2002). The results were considered statistically significant at $p<0.05$. The changes in gene expression levels among chickens treated with low dose (25 µg) or high (50 µg) dose of class B CpG ODN in addition to non-ODN were expressed as a fold change relative to the PBS control treatment group.

Results

To examine the stimulatory effects of class B CpG ODN delivered by intraocular and subcutaneously route on peripheral blood cells, TLR21 IFN-γ and IL-1β mRNA expression levels were quantified. The kinetics of expression of chicken TLR21, were measured upon stimulation. The TLR21 mRNA expression levels were significantly upregulated 3 h poststimulation with a high and low dose of class B CpG ODN after subcutaneous administration in comparison to those found in the control group ($p<0.001$) and to those observed in the non-CpG ODN ($p<0.05$) group (Fig. 1A). In the second experiment, TLR21 mRNA expression levels were upregulated (but not significantly) 3 h post-stimulation with a high dose of class B CpG ODN and 72h post-stimulation with a low dose of class B CpG ODN after intraocular administration (Fig. 1B). The TLR21 mRNA expression was significantly decreased 72 h post intraocular stimulation with high dose of class B CpG ODN (Fig. 1B).

The IL-1β mRNA expression levels were significantly upregulated 3 and 72 h after subcutaneous stimulation with a high dose of class B CpG ODN in comparison to those found in the control and non-CpG ODN ($p<0.001$) groups (Fig. 1C). After intraocular administration, the IL-1β mRNA expression was significantly upregulated 6 h and in later time points in every ODN group. The highest value was observed at 24 h post-stimulation with high class B CpG ODN, however, this value was not statistically significant in comparison to non-CpG ODNs ($p=0.182$) (Fig. 1D).

The IFN-γ mRNA expression was significantly upregulated 12 h after subcutaneous stimulation with a high and low dose of class B CpG ODN in comparison to that observed in the control ($p<0.05$) and non-CpG ODN ($p<0.01$) groups (Fig. 1E). After

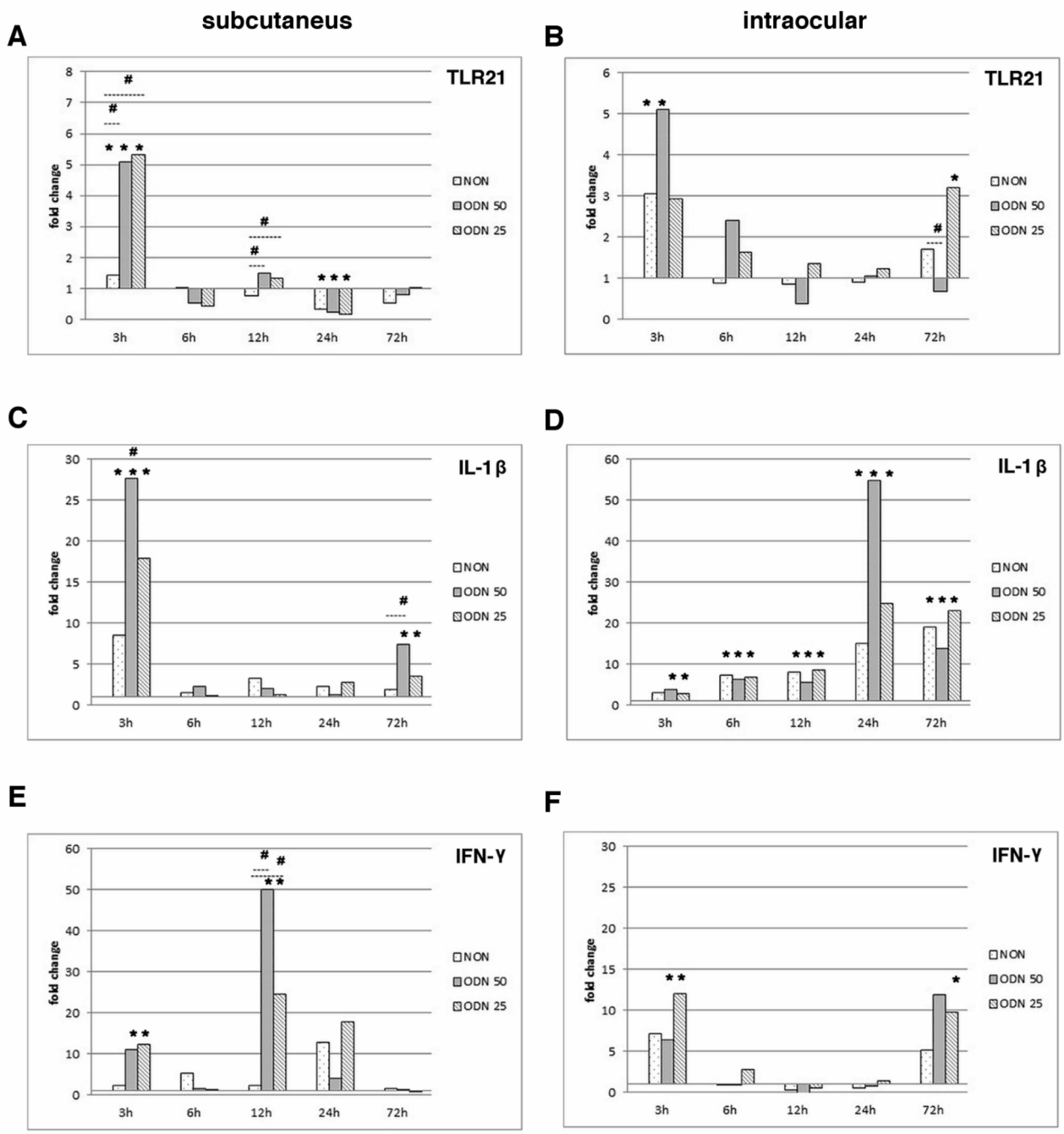

Fig. 1. The relative fold change of mRNA TLR21 (**A** and **B**) IL-1β (**C** and **D**) and IFN-γ (**E** and **F**) in peripheral blood at 3, 12, 24, and 72 hours after the subcutaneous (**A, C** and **E**) and intraocular (**B, D** and **F**) administration of ODNs. The values from PBS control group were set as 1. NON: non-CpG ODN, ODN50: 50 μg/chick of class B ODN CpG: ODN25: 25 μg/chick of class B ODN CpG. The * indicates a statistically significant difference ($p<0.05$) from PBS control. The # indicates a statistically significant difference ($p<0.05$) from nonODN.

intraocular administration the IFN-γ mRNA expression was significantly upregulated 3h and 72h after administration with a low dose CpG ODN and at 3h after administration of a high dose CpG ODN compared to that determined in the PBS control ($p<0.05$) (Fig. 1F).

Discussion

In mammals the umethylated CpG motifs were shown to activate innate and adaptive immune responses by mediating cytokine release from different cell population as well as direct activation of B cells

(Krieg et al. 1995, Klinman et al. 1996). Then, it was shown that this activation is mediated mainly by TLR9 stimaulation (Bauer et al. 2001). In contrast to mammals, the functional counterpart of TLR9, which recognise CpG ODN, in the chicken is TLR21 (Brownlie et al. 2009, Keestra et al. 2010).

In this study we found that the mRNA TLR21 gene expression in peripheral blood was significantly higher 3 h after stimulation with class B CpG ODN administered parenterally, independently of the dose tested. This result is a proof of principle that the CpG ODN used in the study were able to elicit TLR21 signalling. Most likely, the ODNs are taken by immune cells, which later either migrate back to blood in order to reach lymphatic organs for antigen presentation, or secrete the cytokines that alter the TLR21 gene expression in the circulating cells (Jakob et al. 1998, Ban et al. 2000). Interestingly, we found the second smaller pick 12 h post-stimulation, which might reflect those different mechanisms of signalling (Bonizzi and Karin 2004).

Contrary to the results obtained with parenteral administration, after the itraocular application of the ODNs the slightly diminished expression of TLR mRNA was observed 72 h post-stimulation with a high dose of CpG ODN. This may indicate that application of the CpG ODN in the eye does not trigger overexpression of the TLR receptor of other cells in blood. This may be due to the age of the birds, and maturation of the mucosal associated lymphoid tissue and Harderian gland. Indeed, when the cells collected from the Harderian gland in 5week old birds were stimulated with class B CpG ODN, the increase in mRNA expression of TLR21 and IFN-γ was observed 1h after the application. However, when the same cells from 12-week old birds were used, there was no statistical difference in mRNA expression of those molecules (Chrząstek et al. 2014).

The stimulatory effect through TLR receptor includes cytokines secretion, which leads to elicitation of the innate and adaptive immune response (Brownlie et al. 2009, Hung et al. 2011). Additionally, it was previously shown that stimulation of chicken immune cells through TLR21 by class B ODN CpG drives production of Th1 promoting inflammatory cytokine IFN-γ (Patel et al. 2008, Bhat et al. 2010, St Paul et al. 2012). These effects of CpG ODN depend on doses used and differ in time. For instant, Patel et al. (2008) have demonstrated that intramuscular administration of the CpG ODN upregulates IFN-γ mRNA expression in chicken spleen cells at 3 h post-application, then declined to its basal level at 12 h post-application and then increased once again in later time point. In our experiment we also observed that when administered subcutaneously, the CpG ODNs, of a low and high dose, induced upregulation of the IFN-γ mRNA at 12 h after administration.

The CpG ODN has also a capacity to trigger pro-inflammatory cytokine production such as IL-1β (Takeda et al. 2003, He et al. 2003, Patel et al. 2008). Based on our findings, it appears that class B CpG ODN given subcutaneously can upregulate IL-1β mRNA gene expression at early (3 h) and late (72 h) time point after stimulation. Moreover, the class B CpG ODN administered parenterally possessed this immunostimulatory effects only when given at high doses (50 μg), while the low dose did not trigger IL-1β mRNA gene expression. In previous studies Patel et al. (2008) also have shown that after intramuscular administration, spleen and Bursa Fabrici cells respond to CpG ODN stimulation by upregulation IL-1β mRNA gene expression at early and late time point. Interestingly, in this study after intraocular administration the fold increase in transcription of IL-1β was observed in all read-outs with the pick at 24 h post-application, albeit without statistical difference. One can speculate that the IL-1β response after TLR stimulation would be observed only locally, in the eye, but recent *in vitro* studies indicate that it was not the case (Chrząstek et al. 2014).

Previously, it was suggested, that class B CpG ODN, administered by the eye-drop route, have the potential to act as mucosal vaccine adjuvants (Hikono et al. 2013). The ability to induce a potent Th1-type immune response is of considerable importance, because in the infection with various pathogens, cell-mediated immunity is correlated with protection (Patel et al. 2008, Taghavi et al. 2008).

Our study revealed that intraocular application of the class B CpG ODN did not trigger the same response, as when administered parenterally. The reason might be that the class B CpG ODN encounters different cells on the application site. After subcutaneous or intramuscular administration the ODNs are most likely sensed by antigen presenting cells, dendritic cells and macrophages (Jakob et al. 1998, Ban et al. 2000). In contrast, the Harderian gland consists mainly of B cells, plasmatic cells, T cells and scares macrophages (Śmiałek et al. 2011). Another explanation for week triggering of mRNA synthesis of TLR21, IFN-γ and IL-1β in peripheral blood might be that the trigger from TLR21 in mucosal associated lymphoid tissue needs an antigen co-stimulant. Recently, it was shown that the vaccination with nanoparticle containing CpG adjuvant and the antigen induced better innate and adaptive cellular response that CpG alone in mice model (Ilyinskii et al. 2014).

In summary, our findings show that class B CpG ODN delivered by subcutaneous route induces strong

expression of TLR21, IFN-γ and IL-1β in peripheral blood. However, the intraocular application did not elicit similar results. Many experiments have shown previously the adjuvant effect of the class B CpG ODN, therefore it is important to investigate the mode of their action in developing immunity in chicken.

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