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Original article

Impact of CpG oligodeoxynucleotide stimulation on percentage of T and B cells in chicken

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Abstract

TLR stimulation in chickens has been shown to play a role in the initiation and regulation of innate and adaptive immune responses. The aim of this study was to use flow cytometry to establish the percentage of T and B subset in blood and lymphoid organs in chicks after CpG oligodeoxynucleotide (ODN) stimulation. It was demonstrated that the percentages of $CD3^+$, $CD4^+$, $TCR\gamma\,\delta^+$ cells and $Bu\text{-}1^+$MHC class II^+ cells in blood 24 h post-injection were significantly higher than in the control groups. It was also shown that the percentages of $CD3^+$ and $CD4^+$ cells in the spleen at 48 h post-injection were significantly higher than in control groups. The percentage of $Bu\text{-}1^+$ cells in the bursa of Fabricius after CpG ODN stimulation (98.38 ± 0.84) was significantly higher than that found in the non-CpG ODN control group (94.54 ± 2.51) ($p \leq 0.05$). The results indicate that class B CpG ODN increases the percentage of both T (especially $CD4^+$ cells) and B cells.

Key words: ODN CpG, flow cytometry, poultry

Introduction

Toll like receptors (TLR) are responsible for the recognition of conserved motifs found on bacteria, fungi, parasites and viruses known as pathogen associated molecular patterns (PAMPS). Bacterial DNA has long been known as one of the key immunostimulatory PAMPS. In mammals, TLR 9 recognizes bacterial DNA as well as unmethylated CpG DNA motifs and viral nucleic acids (Akira and Takeda 2004). The counterpart of TLR 9 in chicken is TLR 21 (Brownlie et al. 2009, Keestra et al. 2010). CpG ODNs are utilized for a variety of therapeutic purposes. Gomis et al. (2003, 2004) demonstrated that prophylactic treatment of chickens with class B CpG ODN enhanced host immunity to numerous pathogens such as *Escherichia coli*. A number of reports regarding the effects of ODNs on chicken cells cultured *in vitro* (He et al. 2003, 2006, Schwarz et al. 2007) or as a vaccine adjuvant (Linghua et al. 2007, Zhang et al. 2008) have been published. However, little information is available about DNA induced stimulation of chicken leukocytes *in vivo*. Therefore, it was of interest to estimate the percentage of lymphocytes in peripheral blood and lymphatic organs (bursa of Fabricius and spleen) after class B CpG ODN stimulation.

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Materials and Methods

Animals

Twenty four 4-week-old healthy Hubbard Flex breeder male chicks were raised in cages. The housing area was scrubbed and steam-cleaned before the birds' arrival. Lighting and ventilation were identical for all the chickens. All birds were kept in laboratory conditions. Water and commercial feed were available ad libitum. The experiment was conducted with the consent of the Local Ethical Committee for Animal Experiments.

TLR ligands

The synthetic class B CpG ODN ^{2007} and non ODN (Gomis et al. 2003) were purchased from TIB Molbil, Synntheselabor mbH (Berlin, Germany), both possessing a phosphorothioate backbone. All the ligands used were re-suspended in sterile phosphate buffered saline (PBS pH 7.4).

Experimental design

The chickens were allocated to three groups of eight animals each. The experimental group (95.42 g ± 3.95 BW) received a single subcutaneous dose of 50 µg CpG ODN per bird on the first day of the experiment, while the two control groups received 50 µg of non-CpG ODNs or PBS. Sampling was done at 24 and 48 h post-injection. At the first time point, peripheral blood samples (1.0 mL heparinized blood) for immunophenotyping were collected without sacrificing the chickens. Then, at 48 h post-injection, 24 of the animals were euthanized, and the spleens and bursas of Fabricius were subjected to flow cytometric analysis.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) to be used for the flow cytometric phenotypings were purified as described previously by Stenzel et al. (2011). Aliquots of lymphocytes were then incubated with different mouse anti chicken monoclonal antibodies: R-PE- CD3 (clone CT-3), FITC- CD4 (clone CT-4), R-PE-CD8α (clone CT-8), FITC-Bu-1 (clone AV20), RPE- MHCII (clone 2G11), FITC-TCRγδ (clone TCR 1) obtained from Southern Biotech (Birmingham, AL, USA) for 30 min, and then washed three times with PBS (pH=7.4). Chicken PBMC were double stained with: mouse anti-chicken CD4-FITC and mouse anti-chicken CD8α-R-P, mouse anti-chicken Bu-1-FITC and mouse anti-chicken MHC II-R-PE. In addition, PMBC were single stained with CD3-R-PE and TCRγδ – FITC.

Fresh bursas and spleen samples from all birds were collected and immediately homogenized. The bursa and spleen cells were prepared as described previously (Chrząstek et al. 2011, Stenzel et al. 2011). For immunophenotypic analyses, aliquots of bursa lymphocytes ($1x10^6$cells/ml) were incubated with FITC-conjugated Bu-1 monoclonal antibody (Clone AV 20, chB6) (SouthernBiotech, Birmingham, AL, USA), in PBS for 30 min, and then washed three times with PBS (pH 7.4). This antibody recognizes a monomorphic determinant on the Bu-1 B cell associated alloantigens of both the RPL 6 (Bu-1a) and 7 (Bu-1b) lines of inbred chickens (Rothwell et al. 1996). Instead, aliquots of spleen lymphocytes ($1x10^6$cells/ml) were incubated with RPE-conjugated CD3, FITC- CD4, RPE-CD8α, FITC-Bu-1, or R-PE-Ia (clone CIa-I) monoclonal antibodies (SouthernBiotech, Birmingham, AL, USA) in PBS for 30 min, and then washed three times with PBS (pH 7.4). The chicken Ia antigen is expressed preferentially on B cells, however it can also be demonstrated on mitogen-activated T cells (Sachs 1976, Evert et al. 1984). Spleen lymphocytes were double stained with mouse anti-chicken CD4-FITC and mouse anti-chicken CD8α-R-PE, mouse anti-chicken CD3-R-PE and mouse anti chicken Ia-R-PE. In addition, spleen lymphocytes were single stained with Bu-1-FITC.

All samples were analyzed by flow cytometry (Becton-Dickinson Immunocytometry Systems, San Jose, California, USA). For each sample, 20,000 gated cells were acquired, and the percentage of cells in the population was determined using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Statistical analysis

The significance of differences between the results obtained was obtained using Student's t-test (Statistica 9.0 software, StatSoft Polska Sp. z o.o., Kraków, Poland). Significance was defined as $p \leq 0.05$ or $p \leq 0.001$.

Results

After FCM and statistical analysis, there were generally no statistically significant differences be-

Table 1. Results of FCM blood analysis and statistical analysis.

Treatment	N	Cells (%)				
		$CD8^+$	$CD4^+$	$CD3^=$	$TCR\gamma\delta^+$	$Bu\text{-}1^+MHC\ II^+$
		mean ± SD	mean ± SD	mean ± SD	Mean ± SD	mean ± SD
Control (PBS)	8	2.82 ± 1.24	10.38 ± 1.58	15.26 ± 3.31	3.28 ± 0.50	2.73 ± 0.77
ODN CpG	8	3.64 ± 1.15	13.58 ± 2.91*	21.56 ± 4.13**	5.42 ± 0.92*	3.22 ± 0.83*
Non ODN CpG	8	3.49 ± 1.22	10.33 ± 1.73	15.34 ± 1.81	3.88 ± 1.02	2.38 ± 0.51

Results were considered statistically significant if $p \leq 0.05$ (*) or $p \leq 0.001$ (**).

Table 2. Results of FCM spleen cells analysis and statistical analysis.

Treatment	N	Cells (%)					
		$CD8^+$	$CD4^+$	$CD3^+\ Ia^-$	$CD3^+\ Ia^+$	$Ia^+\ CD3^-$	$Bu\text{-}1^+$
		mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
Control (PBS)	8	22.68 ± 2.48	23.28 ± 2.59	53.51 ± 1.27	5.03 ± 1.89	33.44 ± 3.66	29.22 ± 1.91
ODN CpG	8	24.86 ± 1.91	27.53 ± 2.19**	58.65 ± 1.44**	5.60 ± 1.88	32.71 ± 3.51	33.9 ± 1.88
Non ODN CpG	8	23.09 ± 2.29	21.75 ± 2.07	53.9 ± 1.42	4.47 ± 1.15	31.92 ± 2.28	32.17 ± 1.58

Results were considered statistically significant if $p \leq 0.001$ (**).

tween the non-CpG treated group and the PBS treated control group.

The percentages of lymphocytes in peripheral blood (24 h post injection of CpG ODN) are shown in Table 1. It was demonstrated that the percentage of $CD3^+$ cells in blood following CpG ODN stimulation (21.56 ± 4.13) was significantly higher than that found in the non-CpG ODN group (15.34 ± 1.81) ($p \leq 0.001$). After CpG ODN stimulation (13.58 ± 2.91), the percentage of $CD4^+$ cells was also significantly higher in comparison to the non-CpG ODN group ($p \leq 0.05$). The percentage of $TCR\gamma\delta^+$ cells in blood after CpG ODN stimulation (5.42 ± 0.92) was significantly higher than that found in the non-CpG ODN group ($p \leq 0.05$). The percentage of B lymphocytes (Bu-1+MHCII+) was also significantly higher in the CpG ODN group ($p \leq 0.05$).

The percentages of lymphocytes in the spleen 48 h post injection of CpG ODN are shown in Table 2. It was demonstrated that the percentage of $CD3^+$ cells in the spleen after CpG ODN stimulation (58.65 ± 1.44) was significantly higher than that in the non-CpG ODN group (53.9 ± 1.42) ($p \leq 0.001$). Following CpG ODN stimulation (27.53 ± 2.19), the percentage of $CD4^+$ cells was also significantly higher in comparison to the non-CpG ODN group ($p \leq 0.001$).

The percentages of $Bu\text{-}1^+$ cells in bursas following CpG ODN stimulation (98.38 ± 0.84) was significantly higher than that found in the non-CpG ODN group (94.54 ± 2.51) ($p \leq 0.05$).

Discussion

Recognition of PAMPS by TLRs triggers innate host responses, promoting cytokine expression, which leads to the elicitation of innate and adaptive immune responses (Medzhitov 2001). Class B ODNs is strongly immunostimulatory for B cells (Krug et al. 2001). Wattrang (2009) has shown that Bu-1 enriched cells were able to proliferate upon stimulation with class B ODNs *in vitro*. In this study, it was demonstrated that Class B CpG ODNs increase the percentage of $Bu\text{-}1^+MHC\ class\ II^+$ cells in peripheral blood and $Bu\text{-}1^+$ cells in the bursa of Fabricius. Additionally, Patel et al. (2008) have shown that stimulation by Class B CpG ODNs up-regulate IL-10 in the bursa of Fabricius. It is known that this cytokine triggers B cell proliferation and leads to higher expression of MHC class II molecules. It can be inferred that a higher percentage of Bu-1 cells is connected to stimulation through the B cell receptor, as well as an effect of cytokine production, or both which is more probable. Therefore, the increased percentage of B cells in blood might be connected with the release of higher quantities of B cells from the bursa of Fabricius, which enter the circulation after DNA injection. It was also demonstrated that CpG-ODN treatment up-regulates genes that are responsible for a Th1-type immune response in the spleens of newly hatched chickens (Patel et al. 2008), and that CpG ODNs up-regulate IL-6 in the spleen (Jenkins et al. 2009).

This might be in accord with the higher percentage of $CD4^+$ cells observed in the present study. However, the additional studies are needed to confirm this proposal.

In summary, our study provides new information which indicates that the synthetic CpG ODN 2007 has the capacity to increase the percentage of circulating lymphocyte populations, as well as lymphocyte in lymphoid organs. The knowledge concerning ODN CpG treatment will lead in turn to new opportunities for their use in chicken.

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