CpG-oligodeoxynucleotide-stimulated chicken heterophil degranulation is serum cofactor and cell surface receptor dependent

Haiqi Hea,*, Virginia K. Lowryb, Pamela J. Ferroc, Michael H. Kogutb

a USDA, ARS, Southern Plains Agricultural Research Center, 2881 F and B Road, College Station, TX 77845, USA
b Department of Veterinary Anatomy and Public Health, College of Veterinary Medicine, Texas A and M University, College Station, TX 77843, USA
c Department of Poultry Sciences, Texas A and M University, College Station, TX 77843, USA

Received 26 April 2004; revised 30 June 2004; accepted 12 July 2004
Available online 28 August 2004

Abstract

Synthetic oligodeoxynucleotide containing unmethylated CpG motif (CpG-ODN) is immune stimulatory to chicken heterophils. Recognition of CpG-ODN by chicken heterophils leads to the mobilization and release of granules. This CpG-ODN-induced heterophil degranulation was chicken serum (CS)-dependent. Heat-denaturation and membrane filtration of CS revealed that the active serum cofactor(s) was likely a protein in nature with a molecule mass within 50,000 to 100,000. This serum cofactor(s) was heat-resistant at 56 °C for 1 h. The involvement of a cell surface receptor in recognition of CpG-ODN was also demonstrated by (1) trypsin treatment of the heterophils abrogated the degranulation response and (2) CpG-ODN-induced heterophil degranulation was sensitive to the inhibition of Clathrin-dependent endocytosis. In addition, among various microbial agonists, including CpG-ODN, lipopolysaccharide, lipoteichoic acid, phorbol myristate acetate, and formalin-killed Salmonella enteritidis, CpG-ODN was the only agonist that displayed serum-dependent induction of degranulation in chicken heterophils. This is the first report that shows serum-dependent activation of leukocytes by CpG-ODN.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: CpG-ODN; TLR9; Serum cofactor; Degranulation; Innate immune response; Heterophil; Neutrophil; Chicken

1. Introduction

Heterophils are polymorphonuclear (PMN) leukocytes in poultry and functionally equivalent to mammalian neutrophils. They are the second most numerous cell types in circulating blood of most birds [1]. As a major component of cellular innate immunity, heterophils play a critical role as the first line of defense against microbial infection. In poultry, these granulocytic phagocytes are rapidly recruited to the site of infection, where they engage in phagocytosis and killing of pathogens [2,3]. Activation of heterophils has been shown to be critical in protecting neonatal chickens from systemic
infection of *Salmonella* and *Salmonella*-induced mortality [3,4]. This heterophil-mediated immunity was associated with recruitment of large numbers of heterophils to the site of bacterial invasion and an increased number of peripheral blood heterophils [5,6]. Heterophils, like mammalian neutrophils, respond to microbial stimulation by producing toxic reactive oxygen species and releasing bactericidal substances and proteolytic enzymes in the process of degranulation [1–3,7–9].

DNA from bacteria, but not vertebrates, is a powerful stimulator of the innate immune system. The immune stimulatory activities of bacterial DNA are attributed to the presence of a high frequency of an unmethylated CpG dinucleotide motif, which is an unmethylated cytosine followed by guanosine (CpG) dinucleotide flanked by certain nucleotides [10]. It has been well documented that bacterial DNA and synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG-dinucleotides (CpG-ODN) activate B-lymphocyte and innate immune cells (macrophage, dendritic cells, and natural killer cells) to secret cytokines [interleukin-1β (IL-1β), IL-6, IL-12, IL-18, tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), and IFN-γ] and to promote adaptive immune responses [10]. Recognition of CpG-ODN and signaling of cell activation are mediated by the Toll-like receptor 9 (TLR9) [11,12]. Interaction of CpG-ODN with TLR9 occurs exclusively in the intracellular compartment [13]. Internalization by endocytosis and subsequent endosomal maturation is therefore the prerequisite for CpG-ODN activity [14].

Despite the fact that neutrophils play an important role in innate immunity as mediators of immune recognition and destruction of microbial pathogens, there is a lack of research on neutrophil responses to CpG-ODN stimulation [15–17]. More recently, studies on immune stimulatory activities of CpG-ODN in avians have begun to emerge [18–22]. We have previously demonstrated the CpG motif-dependent induction of nitric oxide (NO) production in an avian macrophage cell line (HD11) by synthetic CpG-ODN [19]. Further studies demonstrated that induction of NO in HD11 cells by CpG-ODN requires clathrin-dependent endocytosis and endosomal maturation [20]. However, immune stimulatory activity of CpG-ODN in chicken heterophils has not been reported. In this study, we focused on activation of heterophil degranulation by a previously identified avian immune stimulatory CpG-ODN [19,20]. Our study shows that activation of heterophil degranulation by CpG-ODN is dependent on the presence of chicken serum (CS). In the presence of CS, CpG-ODN induces dose-dependent activation of heterophil degranulation.

### 2. Materials and methods

#### 2.1. Reagents

The nuclease-resistant phosphorothioate ODNs were purchased from Integrated DNA Technologies (Coralville, IA, USA) and further purified by ethanol precipitation. ODNs were dissolved in sterile phosphate-buffered saline (PBS, pH 7.2) at a concentration of 1 mg/ml. The sequences of synthetic ODNs used in the present study were: CpG-ODN#17, GTC GTT GTC GTT GTC GTT; and a non-CpG containing control ODN (nCpG-ODN), CCA TGG CCA TGG CCA TGG [19]. Lipopolysaccharide (LPS, from *Salmonella enteritidis*), lipoteichoic acid (LTA, from *Staphylococcus aureus*), phorbol myristate acetate (PMA), CS and fetal bovine serum (FBS), RPMI-1640, Hank’s balanced salt solution (HBSS), Histopaque, monodansylcadaverine (MDC), and chloroquine were purchased from Sigma (St Louis, MO, USA).

#### 2.2. Animals

Leghorn chickens (2–4 day old), obtained from a local commercial hatchery (Hy-Line International, Bryan, TX, USA), were used as blood donors for heterophil isolation. Chickens were kept in a controlled environment with ad libitum access to water and feed. The diet was a balanced and un-medicated corn-soybean ration [23].

#### 2.3. Isolation of heterophils

Collection of peripheral blood and heterophil isolation were conducted as previously described [8]. Briefly, peripheral blood from approximately 50 chicks was pooled, mixed with 1% methylcellulose...
(1:1 v/v), and centrifuged at 25 × g (rcf) for 15 min. The supernatant was removed, diluted with Ca2+- and Mg2+-free Hanks balanced salt solution, carefully layered onto a discontinuous Histopaque gradient (specific gravity 1.077/1.119) in 50-ml conical centrifuge tubes, and centrifuged at 250 × g for 60 min. The heterophils, below the Histopaque 1.077/1.119 interface, were collected, washed, and re-suspended in RPMI-1640. Heterophils were counted and kept on ice until used.

2.4. Preparation of formalin-killed S. enteritidis (FKSE)

A primary poultry isolate of S. enteritidis (NVSL#97-11771, National Veterinary Services Laboratory, Ames, IA, USA) was used in this study. Bacteria were inoculated and cultured overnight in tryptic soy broth. Formalin-killed bacteria were prepared by incubation of bacteria in 1% formalin solution at 4 °C for 24 h followed by washing four times with PBS to remove the formalin, and stored at 4 °C until used.

2.5. Degranulation assay

Heterophil degranulation was measured by quantifying β-glucuronidase activity [24] in culture medium following stimulation of heterophils (8 × 10⁶/ml) with CpG-OND or other agonists in the presence or absence of CS for 60 min on a rocker platform at 39 °C in a 5% CO₂ incubator. In the inhibition assay, heterophils (8 × 10⁶/ml) were treated with or without an inhibitor for 30 min prior to stimulation with CpG-ODN. After incubation, the cells were pelleted by centrifugation at 250 × g (rcf) for 10 min at 4 °C and supernatants were collected for the assay. An aliquot of 25 µl supernatant was incubated with 50 µl of freshly prepared substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide, 0.1% Triton X-100 in 0.1 M sodium acetate buffer) for 4 h at 41 °C. The reaction was stopped by adding 200 µl of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone was measured fluorimetrically (355/460 nm) using an f_max fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

2.6. Data analysis

Data are means and standard deviations of three independent experiments with three to five-replicates each. Statistical difference was determined at the level of p < 0.05 by Student’s t-test using SigmaStat software (Jandel Corp, San Rafael, CA, USA).

3. Results

3.1. Serum dependence of CpG-ODN induced heterophil degranulation

The effects of CpG-ODN on heterophil degranulation are dependent on the presence of CS. In the absence of CS, incubation of heterophils with CpG-ODN reduced release of β-glucuronidase even below the level of non-stimulated spontaneous degranulation (Fig. 1A). In the presence of CS, CpG-ODN stimulated dose-dependent degranulation by heterophils (Fig. 1B). Likewise, increasing concentration of CS in the medium enhanced CpG-ODN-stimulated degranulation in heterophils (Fig. 1C). Additionally, ODN containing immunostimulatory CpG motifs was significantly more effective than the control ODN without CpG motif (nCpG-ODN) in the induction of heterophil degranulation (Fig. 1D). Pre-incubation of heterophils with CpG-ODN followed by washing did not retain CpG-ODN stimulatory activity for degranulation when serum was subsequently added to the reaction (data not shown). This indicates that serum components were required to initiate CpG-ODN signal leading to heterophil degranulation.

3.2. Comparison of agonist, CpG-ODN, LPS, LTA, PMA, and FKSE stimulation on heterophil degranulation

To determine if this serum-dependent heterophil degranulation is a CpG-ODN specific phenomenon, degranulation activities were compared when heterophils were stimulated with various agonists such as CpG-ODN, LPS, LTA, PMA, and FKSE (Fig. 2). The results show that LPS and LTA do not induce heterophil degranulation, whereas, PMA and FKSE stimulate heterophil degranulation regardless of
the presence of serum. Only CpG-ODN showed the serum dependence in its ability to induce the heterophil degranulation.

3.3. Effects of CS filtrates from different molecule-weight-cut-off membranes on CpG-ODN induced degranulation

To estimate the molecule mass of the serum active component, CS was fractionated with Centriprep with 10,000, 50,000 and 100,000 molecule weight cut-off (MWCO) membranes (Amicon, Beverly, MA) by centrifugation at 1800×g (rcf). The filtrates were tested for their ability to mediate CpG-ODN induced degranulation. Our results indicate that serum active component resides in the molecule mass range from 50,000 to 100,000, since the 100,000 MWCO filtrate retained 100% activity for CpG-ODN induced degranulation.

Fig. 1. Serum dependence of CpG-ODN induced heterophil degranulation. (A) Heterophils (8×10⁶/ml) were stimulated with 5 μg/ml of CpG-ODN with or without CS (5%). Supernatants were harvested after the indicated length of incubation (at 39 °C and 5% CO₂) and β-glucuronidase activity was measured. (B) Heterophil stimulated with CpG-ODN at various concentrations for 60 min. (C) Heterophil stimulated with 5 μg/ml of CpG-ODN at various concentrations (%) of CS for 60 min. (D) Comparison of heterophil degranulation stimulated by CpG-ODN or nCpG-ODN for 60 min with or without CS (5%). *p<0.05 compared to the control.

Fig. 2. Comparison of heterophil degranulation stimulated by various agonists (CpG-ODN, LPS, LTA, PMA, and FKSE) with or without CS. Heterophils were stimulated with CpG-ODN (5 μg/ml), LPS (10 μg/ml), LTA (10 μg/ml), PMA (1 μg/ml), or FKSE (equivalent to 10⁸ CFU of live SE) for 60 min with or without CS (5%). Heterophils in the controls were unstimulated. *p<0.05 compared to the control.
degranulation, whereas, fractions between 10,000 and 50,000 MWCO completely lost their activity (Fig. 3).

3.4. Effect of heat denaturation of CS on CpG-ODN induced degranulation

The heat-denaturation of CS at various temperatures was used to determine if the complement cascade was involved in mediating CpG-ODN induced heterophil degranulation. Heating CS at 56 °C on a waterbath for 60 min, which inactivates the complement system, did not reduce the activity of CS in CpG-ODN stimulated degranulation. However, activity was lost when CS was heated at 75 and 95 °C for 30 min (Fig. 4). These results indicate that the CpG-ODN induced degranulation was probably mediated by protein components of the serum other than complement system.

3.5. Comparison of effectiveness of chicken and bovine sera in CpG-ODN induce heterophil degranulation

Since CS was shown to be necessary for CpG-ODN to induce heterophil degranulation, we compared the effectiveness of CS and FBS for CpG-ODN stimulated degranulation (Fig. 5). The results indicate that FBS was also able to mediate CpG-ODN induced degranulation, however, it was significantly less effective compared to the CS at the same concentration. This observation indicates that there is certain cross-species activity of the serum cofactor in mediating CpG-ODN induced degranulation.

3.6. Involvement of cell surface receptor in CpG-ODN induced heterophil degranulation

Intracellular localization of TLR9 [14] suggests CpG-ODN must be internalized in order to activate cellular function. This internalization of CpG-ODN is most likely receptor mediated endocytosis. However, a cell surface CpG-ODN binding protein or a receptor has not been identified. To demonstrate...
the involvement of a receptor in CpG-ODN-mediated stimulatory activities, the heterophils were incubated with 0.05% trypsin ATv solution prior to stimulation with CpG-ODN. Our results show that treating heterophils with trypsin proteinase for 30 min completely abolished CpG-ODN induced degranulation (Fig. 6).

3.7. Roles of clathrin-dependent endocytosis and endosomal maturation in CpG-ODN induced heterophil degranulation

Clathrin-dependent endocytosis is a well characterized mechanism and is utilized by many receptor mediated endocytic pathways [25]. MDC is a selective inhibitor for clathrin-dependent endocytosis [26–28]. Pretreatment of heterophils with MDC resulted in dose-dependent inhibition of CpG-ODN induced degranulation (Fig. 7A). These results indicate the clathrin-dependent endocytic pathway was involved in CpG-ODN signaling. Our results further show that subsequent endosomal maturation was critical for CpG-ODN stimulated heterophil degranulation. Increasing concentration of chloroquine, an endosomal maturation inhibitor, in the media attenuated the stimulatory effect of CpG-ODN on heterophil degranulation (Fig. 7B). This result confirms the essential role of endosomal maturation/acidification in CpG-ODN mediated cellular signaling.

4. Discussion

The innate immune system has developed and been conserved during the course of evolution for rapid recognition and response to invading pathogens through recognition of pathogen associated molecule patterns (PAMPs). One example of such recognition of PAMPs is that the vertebrate innate immune system (from fish and birds to mammals) can discriminate both bacterial and viral DNA from self DNA by recognition of unmethylated CpG motifs present in the pathogen’s DNA [10]. CpG DNA therefore is recognized as a danger signal by the host and evokes innate immune responses, which in turn promote the host adaptive immune system to combat potential infection.
Chicken heterophil degranulation plays an important role in host defense and inflammatory activity. Granule proteins, including cationic bactericidal peptides [29], defensins [30], and proteolytic enzyme such as gelatinase B [31], a matrix metalloproteinase, have been identified in heterophils. These granule proteins are prepacked in the granules and released in response to certain microbial agonist stimulation to engage bactericidal activities and aid in heterophils’ migration to the site of infection. Deficiency in granule proteolytic protein has been associated with impaired bacterial killing capacity of neutrophils [32].

The specific interaction of pathogen-associated molecules with soluble and cell-surface located pattern recognition receptors is a prerequisite for initiating host innate immune responses to a pathogen. At least four soluble microbial molecule-binding proteins, including LPS-binding protein (LBP) [33–35], soluble CD14 [33–35], mannose-binding lectin [36], and one of the peptidoglycans recognition proteins (PGRP) [37] have been reported to play significant roles in pattern recognition and modulation of innate immune responses. The roles of LBP and CD14 in activation of Toll-like receptors (TLR2 and TLR4) in the innate immune cells are well documented [33]. The responses of both TLRs are strongly dependent on serum, where LBP and soluble CD14 are the active components [33]. Recognition of CpG-ODN and signaling cell activation are mediated by the Toll-like receptor 9 (TLR9) [11,12]. Interaction of CpG-ODN with TLR9 has been shown to occur exclusively in the intracellular compartment [13]. Internalization by endocytosis and subsequent endosomal maturation is therefore the precondition for CpG-ODN activity [14,20]. However, the initial recognition of CpG-ODN by immune cells is unknown.

Here, we demonstrated that immune recognition of CpG-ODN by chicken heterophils is serum dependent. This recognition of CpG-ODN led to the mobilization and release of heterophil granules in the process of degranulation. The response of heterophil degranulation to CpG-ODN stimulation requires the presence of serum in the medium (Fig. 1A). Removal of CS from the cell culture rendered heterophils unresponsive to CpG-ODN stimulation. Those results indicate that recognition of CpG-ODN by heterophils requires facilitation of serum cofactor(s) to induce signal transduction leading to mobilization and release of granules. CpG-ODN stimulating leukocyte activation has been demonstrated in numerous species. However, this serum-dependent activation of leukocyte by CpG-ODN is the first to be reported. In fact, serum is routinely added to cell culture media when stimulated with CpG-ODN and its effect on CpG-ODN induced activation has not been evaluated [17]. Our results also show that synthetic ODN containing immune stimulatory CpG motif was more effective \( (p \leq 0.05) \) in stimulating heterophil degranulation than ODN containing no CpG motif (Fig. 1D). However, in this study, we did observe an elevated level \( (p \leq 0.05) \) of degranulation when heterophils were stimulated with the control ODN containing no CpG motif in the presence of serum (Fig. 1D). Human neutrophils also have been shown to degranulate in response to CpG-ODN stimulation, however, the response was not serum dependent [16].

Our results indicate that CS contains cofactor(s) which facilitate CpG-ODN recognition by a heterophil cell surface receptor, leading to heterophil activation. Although serum cofactor(s) and receptor(s) for CpG-ODN recognition have not been physically identified, evidence from this study supported the conclusion. First, our results show serum has to be present in the cell culture medium in order for CpG-ODN to induce heterophil degranulation (Fig. 1A). CpG-ODN itself failed to induce any degranulation activity in heterophils. Heterophils appeared unable to bind CpG-ODN without the presence of CS, since heterophils, which were preincubated with CpG-ODN followed by washing to remove CpG-ODN from the medium, displayed little degranulation activity when serum was added back to the culture medium. Secondly, heat denaturation and fractionation of CS demonstrated that the active serum cofactor(s) was most likely proteinaceous with a molecule mass in the range of 50,000–100,000 (Fig. 3). The active serum cofactor(s) was heat-resistant up to 56 °C for 1 h without any loss of activity (Fig. 4), which ruled out the participation of complement cascade. This active serum cofactor also demonstrated cross-activity between species (chicken and bovine) (Fig. 5).
Lastly, recognition of CpG-ODN by cell surface receptor(s) was demonstrated by the loss of degranulation activity when heterophils were treated with trypsin for 30 min prior to CpG-ODN stimulation (Fig. 6). Together, these results suggest that unidentified serum cofactor(s) may act as a CpG-ODN binding factor facilitating interaction of CpG-ODN with cell surface receptor(s) to initiate the process of degranulation. However, we do not know if the TLR9 was involved in the recognition of CpG-ODN at the cell surface and mediated the signal transduction pathway.

Clathrin-mediated endocytosis is a well characterized mechanism which involves many receptor-mediated endocytic pathways [25], therefore, to further demonstrate the involvement of a receptor in mediating CpG-ODN induced heterophil degranulation, we examined clathrin-mediated endocytosis pathway using a selective inhibitor, MDC. The results indicate that the clathrin-dependent endocytic pathway is involved in CpG-ODN signaling due to pretreatment of heterophils with MDC resulted in dose-dependent inhibition to CpG-ODN induced degranulation (Fig. 7A). Furthermore, we showed that subsequent endosomal maturation was important for CpG-ODN stimulated heterophil degranulation. This was demonstrated by the diminished stimulatory effect of CpG-ODN on heterophil degranulation as a result of increased concentration of chloroquine, an endosomal maturation inhibitor, in the media (Fig. 7B). These results support previous reported findings that induction of NO by CpG-ODN in chicken HD11 macrophage cell line is mediated by clathrin-dependent endocytosis and requires endosomal maturation [20].

Additionally, our study demonstrated differential responses of heterophils to various microbial agonists. At the doses examined, heterophils were unresponsive to LPS and LTA stimulation in terms of induction of degranulation regardless of the presence of CS. On the other hand, PMA and FKSE both induced serum-independent degranulation reaction in heterophils. CpG-ODN was the only agonist examined that displayed serum-dependent induction of degranulation in chicken heterophils. These results indicate that differential signaling pathways are probably involved in heterophil activation by these agonists and that effector functions may vary in response to different microbial agonists.

In summary, we have demonstrated the participation of a serum cofactor and a cell surface receptor in CpG-ODN stimulated degranulation of chicken heterophils. These results allow us to hypothesize that the serum cofactor mediates the interaction of the CpG-ODN and possibly the nCpG-ODN as well with the cell surface receptor, where both CpG-ODN and nCpG-ODN are internalized by Clathrin-dependent endocytosis. This endocytosis activity may contribute certain degree of degranulation by heterophils in response to both CpG- and nCpG-ODN stimulation. However, recognition of the internalized CpG-ODN by the TLR9 leads to a greater activation of heterophils which results in significantly higher degranulation activity. Further studies will be conducted to identify and characterize the serum cofactor and the cell surface receptor of the heterophils.

Acknowledgements

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References


