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Chicken mannose-binding lectin function in relation to antibacterial activity towards *Salmonella enterica*

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ABSTRACT

Mannose-binding lectin (MBL) is a C-type serum lectin of importance in innate immunity. Low serum concentrations of MBL have been associated with greater susceptibility to infections. In this study, binding of purified chicken MBL (cMBL) to *Salmonella enterica* subsp. *enterica* (*S. enterica*) serotypes B, C1 and D was investigated by flow cytometry, and *Staphylococcus aureus* (*S. aureus*) was used for comparison. For *S. enterica* the C1 serotypes were the only group to exhibit binding to cMBL. Furthermore, functional studies of the role of cMBL in phagocytosis and complement activation were performed. Spiking with cMBL had a dose-dependent effect on the HD11 phagocytic activity of *S. enterica subsp. enterica* serovar Montevideo, and a more pronounced effect in a carbohydrate competitive assay. This cMBL dose dependency of opsonophagocytic activity by HD11 cells was not observed for *S. aureus*. No difference in complement-dependent bactericidal activity in serum with high or low cMBL concentrations was found for *S.* Montevideo. On the other hand, serum with high concentrations of cMBL exhibited a greater bactericidal activity to *S. aureus* than serum with low concentrations of cMBL. The results presented here emphasise that chicken cMBL exhibits functional similarities with its mammalian counterparts, i.e. playing a role in opsonophagocytosis and complement activation.

Introduction

The C-type lectin, mannose-binding lectin (MBL) acts in the first line of defence against invasion of viral, parasitic and bacterial pathogens. MBL is a serum protein and is primarily produced by hepatocytes (Holmskov et al., 2003). MBL is able to bind to specific sugar patterns, i.e. mannose-rich carbohydrates present on microbial cell surfaces, using its carbohydrate recognition domain (CRD) and thereby acting as a humoral pattern-recognition receptor. First, human MBL can enhance opsonophagocytosis by leukocytes (reviewed by Garred et al., 2009). This also includes enhancement of opsonophagocytosis of necrotic and apoptotic cells after binding to their altered endogenous ligands (Nauta et al., 2003; Ogden et al., 2001). Second, human MBL can activate the complement system via the lectin-dependent pathway. MBL interacts with the mannose-binding associated proteases (MASPs) in order to induce the complement cascade reactions. In humans, MASP-1 promotes transactivation of MASP-2 (Heja et al., 2012), but MBL-MASP-2 complexes have also been shown to activate the complement cascade in the absence of MASP-1 (Ip et al., 2009; Vorup-Jensen et al., 2000). Third, the human MBL/MASP complex has been shown to influence the coagulation pathway, indicating a role for MBL in cell homeostasis and coagulation as well (La Bonte et al., 2012). In humans, MBL deficiency has been associated with increased susceptibility to infections (reviewed by Takahashi, 2011; Heitzeneder et al., 2012).

MBL has been isolated and characterised from several species, among these humans (Kawasaki et al., 1983), mice (Sastry et al., 1995), cattle (Loveless et al., 1989), pigs (Agah et al., 2001), fish (Nikolakopoulou and Zarkadis, 2006) and chickens (Laursen et al.,

Abbreviations: CRD, carbohydrate recognition domain; cMBL, chicken MBL; EDTA, ethylenediaminetetraacetic acid; FSC, forward scatter; GalNAc, N-acetyl galactosamine; GlcNAc, *N*-acetylglucosamine; HKB, heat killed bacteria; IBV, infectious bronchitis virus; L10L, Chickens L10 selected for low serum concentration of MBL; L10H, Chickens L10 selected for high serum concentration of MBL; ManNAc, Mannosamine; MASP, mannose-binding associated protease; MBL, mannose-binding lectin; *P., Pasteurella*; RPM, revolutions per minute; RT, room temperature; *S. aureus*, *Staphylococcus aureus*; *S. enterica*, *Salmonella enterica*; SSC, side scatter; VBS, veronal buffered saline; WT, wild type.

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1995). In correspondence with the human studies, low concentrations of MBL also impact disease severity in chickens. For investigation the effect of MBL on disease severity in chickens, outbred chicken lines have been used to investigate the influence of MBL concentrations during a *Pasteurella* (*P.*) *multocida* infection, and a correlation between low concentrations of MBL and systemic *P. multocida* infection was shown (Schou et al., 2010a). Furthermore, the inbred chicken line L10 has been used as an *in vivo* model (Juul-Madsen et al., 2007) in which an MBL effect was shown in relation to infections with infectious bronchitis virus (IBV) (Juul-Madsen et al., 2007; Kjaerup et al., 2014), *Escherichia coli* (*E. coli*) (Norup et al., 2009), and more recently *Salmonella* subsp. *enterica* serovar Infantis (Ulrich-Lynge et al., 2014).

Only one functioning form of chicken MBL (cMBL) has been described in chickens (Lynch et al., 2005). The order for cMBL binding of monosaccharides is ManNAc > L-fucose > mannose > GlcNAc (Laursen et al., 1995), while for human MBL it is GlcNAc > L-fucose, mannose, mannosamine (ManNAc) > maltose > glucose » *N*-acetyl galactosamine (GalNAc) (Holmskov et al., 1994). In chickens two MASPs and one MAp have been cloned and characterised, MASP-2, -3 and MAp19 (Lynch et al., 2005). A chicken MASP-1 equivalent to human MASP-1 has been identified, but because of the lack of an exon in the chicken MASP-1 gene it is not functional (Lynch et al., 2005). Despite the apparent absence of MASP-1 in chickens, cMBL appears to be able to drive the activation of the complement cascade; this has been demonstrated in a heterologous system by deposition of human C4b on the chicken MBL-MASP complex (Juul-Madsen et al., 2003; Norup and Juul-Madsen, 2007).

In chickens the average cMBL serum concentration is approximately 6 μg/ml based on 14 different chicken lines representing both broilers and layers, and the cMBL concentration in serum ranges from less than 1 to 35 μg/ml or more due to genetic variations (Laursen et al., 1995). Two-three fold upregulation of cMBL has been observed during the acute stages of infection, making it a minor acute phase protein in the chicken (Juul-Madsen et al., 2007; Laursen and Nielsen, 2000; Nielsen et al., 1999).

Serovars of *Salmonella enterica* subsp. *enterica* (*S. enterica*) may cause human food borne salmonellosis, gastro-enteritis and in severe cases death of the patient. It can be subdivided into different serogroups based on repeat units of the lipopolysaccharide chain (designated O-antigen) (CDC, 2013; Rycroft, 2000). The most commonly known and investigated serogroups with zoonotic activity are serogroup B which include *S.* subsp. *enterica* serovar Typhimurium and serogroup D which includes *S.* subsp. *enterica* serovar Enteritidis. However, poultry infections caused by serotypes from the C1 serogroup including *S.* Infantis and *S. subsp. enterica* serovar Montevideo have been emerging during the last decade (EFSA, 2012; Hauser et al., 2012). Furthermore, host-specific serotypes such as *S.* subsp. *enterica* serovar Gallinarum and *S.* subsp. *enterica* serovar Pullorum, both from serogroup B, are of importance as they may cause typhoid-like symptoms sometimes leading to death in poultry (Calenge et al., 2010). A number of *in vitro* studies have been made in relation to human MBL and its interaction with *Salmonella* strains. These studies show that human MBL binds to several serotypes of *S. enterica* especially to the C1 serogroup, the studies have especially focused on *S.* Montevideo, which contains multiple mannose-residues on its O-antigen (Valtonen, 1977; Schweinle et al., 1989; Devyatyarova-Johnson et al., 2000). Differences in the major histocompatibility complex (MHC) have been shown to influence the susceptibility toward *S.* Enteritidis (Schou et al., 2010b) However, it remains unclear if cMBL is able to bind to and/or influence immune responses to any of the more than 2500 serotypes of *S. enterica*.

The main objective of this study was to investigate the binding capacity of cMBL towards wild type (WT) *S. enterica* strains from serogroup B, D and C1 in comparison with *Staphylococcus aureus* (*S. aureus*), and to elucidate the functional properties of cMBL in relation to opsonophagocytosis and complement activation by *in vitro* studies.

Materials and methods

Bacterial strains

A list over the bacterial strains used in this study is shown in Table 1. A range of WT *S. enterica* strains were kindly donated by John Elmerdahl Olsen, Copenhagen University, Denmark. Furthermore, the Gram-positive bacterium *Staphylococcus aureus* (HPA Culture Collections, Public Health England, UK, cat. no. NCTC 06571) was used for comparison in the experiments throughout the study as it has been confirmed to bind strongly to human MBL (Neth et al., 2000).

Growth and preparation of bacteria

S. enterica strains and *S. aureus* were removed from frozen storage at −80 °C and streaked/cultured at 37 °C on LB agar for 16 to 18 h (Sigma-Aldrich Co. LLC., Brøndby, cat. no. L2897). A single colony of each bacterial strain was thereafter cultured in LB broth (Sigma-Aldrich Co. LLC., Brøndby, cat. no. L3022) at 37 °C for 18 h, until $OD_{600\,nm} = 1$. The colony forming units (CFU)/ml of bacteria solution were further validated by plating dilutions on LB agar, followed by incubation at 37 °C for 18 h.

Purification of chicken MBL

For the phagocytic assay, purified cMBL in TBS (10 mM tris, 147 mM NaCl pH 7.4) was used. It was purchased from the Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, Odense. It was purified as previously described (Laursen et al., 1998).

Measurement of serum MBL concentrations

For the phagocytic assay and the bactericidal assay, serum from the chicken line L10 was used. Selective breeding of chickens for low or high serum MBL concentrations has been performed for several generations at our department as published by Juul-Madsen et al. (2007), and the two sublines have been designated, (L10L) for low or (L10H) for high concentrations of serum MBL. The ELISA for measuring the concentration of cMBL was performed as previously described (Juul-Madsen et al., 2007; Juul-Madsen et al., 2003).

Part 1—cMBL binding to S. enterica and S. aureus

Conjugation of fluorochrome to anti-MBL antibody

The anti-MBL antibody (Statens Serum Institut, Copenhagen, Denmark, mouse monoclonal anti-MBL antibody cat. no. HYB 182-01) was labelled with the fluorochrome APC according to the manufacturer's instructions (AbD serotec, Düsseldorf, Germany, LYNX RAPID APC antibody conjugation kit, cat. no. LNK034APC). The conjugated antibody was stored at 4 °C until use.

Detection of cMBL binding to S. enterica and S. aureus

The binding of MBL to 14 *S. enterica* strains from serotypes C1, B and D (Table 1) and *S. aureus* (NCTC 06571) was determined by a flow cytometric procedure, adapted from Devyatyarova-Johnson et al. (2000). First, 50 μl aliquots of the bacterial suspensions containing approximately 1×10^8 CFU/ml were centrifuged at 9600 × *g* for 1 min, and washed with 500 μl veronal buffered saline (VBS) (supplemented with 5 mM CaCl2 and 5 mM MgCl2, (Lonza, Walkersville, USA, cat. no. 12-624E)) (VBS^{2+}). The pellet was resuspended