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Multiple routes of complement activation by *Mycobacterium bovis* BCG

Maria V. Carroll[a,b,*], Nathan Lack[b], Edith Sim[b], Anders Krarup[a], Robert B. Sim[a,b]

[a] MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK
[b] Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK

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ABSTRACT

Mycobacterium tuberculosis is the leading cause of infectious disease in humans in the world. It evades the host immune system by being phagocytosed by macrophages and residing intracellularly. Complement-dependent opsonisation of extracellular mycobacteria may assist them to enter macrophages. This work examines in detail the mechanisms of complement activation by whole mycobacteria using *Mycobacterium bovis* BCG as a model organism. *M. bovis* BCG directly activates the classical, lectin and alternative pathways, resulting in fixation of C3b onto macromolecules of the mycobacterial surface. Investigation into the classical pathway has shown direct binding of human C1q to whole mycobacteria in the absence of antibodies. Most human sera contain IgG and IgM-anti-(*M. bovis* BCG), and pre-incubation with human immunoglobulin enhances C1q binding to the bacteria. Therefore classical pathway activation is both antibody-independent and dependent. The bacteria also activate the alternative pathway in an antibody-independent manner, but Factor H also binds, suggesting some regulation of amplification by this pathway. For the lectin pathway we have demonstrated direct binding of both MBL and L-ficolin from human serum to whole mycobacteria and subsequent MASP2 activation. H-ficolin binding was not observed. No *M. bovis* BCG cell surface or secreted protease appears likely to influence complement activation. Together, these data provide a more detailed analysis of the mechanisms by which *M. bovis* BCG interacts with the complement system.

1. Introduction

Mycobacterium tuberculosis is the leading infectious disease in the world, infecting a third of the world's human population. This bacterium evades the host immune system by residing inside the phagosome of mononuclear phagocytes of the host (Armstrong and Hart, 1975; Clemens and Horwitz, 1995; Sturgill-Koszycki et al., 1996). Prior studies have demonstrated that *M. tuberculosis* can activate complement and entry into the macrophage may be facilitated by interaction with macrophage complement receptors (Ferguson et al., 2004; Ramanathan et al., 1980; Schlesinger, 1993; Schlesinger et al., 1990).

The mycobacterial cell surface is made up of a peptidoglycan layer which covers the lipid bilayer. Covalently attached to the peptidoglycan are arabinogalactans, arabinomannans, glycolipids and mycolic acids which are characteristic of the mycobacterial cell wall (Daffe and Draper, 1998). In addition to the arabinomannan–peptidoglycan layer there is a capsule layer surrounding the mycolates composed mainly of proteins, polysaccharides and lipids. The capsular lipids consist mainly of phospholipids and glycolipids (Daffe and Etienne, 1999) such as trehalose dimycolate (cord factor), which can activate complement (Ramanathan et al., 1980). This complex protective cell wall has important roles in avoidance of killing by the immune system and drug treatments (Besra, 1998).

The complement system is the body's first line of defence against invasion of pathogens. It is made up of three pathways: classical, alternative and lectin pathway, which differ in the stimuli to which they respond. The classical pathway is activated by C1q binding to the complement activator either directly or via bound antibodies. The lectin pathway is activated by the binding of mannose-binding lectin (MBL) or ficolins to the complement activator (Matsushita et al., 2000; Matsushita and Fujita, 1992, 2001). The alternative pathway is activated by a wide range of targets, but it does not have a key initiator like C1q or MBL. Activation of the alternative pathway is based on the continuous spontaneous hydrolysis of C3 to C3(H_2O). Complement activation by any pathway results in the formation of a C3 convertase and the deposition of C3b on target surfaces to trigger opsonisation and other immunoregulatory functions. A major regulator of complement activation is Factor H (FH), which acts as a cofactor for factor I during the cleavage of C3b to iC3b (Sim et al., 1993; Whaley and Ruddy, 1976a).

Pathogenesis by microorganisms relies on their ability to evade killing by the host immune system. Many bacteria have evolved mechanisms to avoid the immune system by down-regulating com-

* Corresponding author at: Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK. Tel.: +44 186 527 1595.
E-mail address: maria.carroll@bioch.ox.ac.uk (M.V. Carroll).

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plement activation by mimicking complement inhibitors or binding host complement inhibitors, such as FH. Other bacteria produce proteases to destroy complement proteins and hence stop the complement cascade such as *Salmonella enterica* and *Porphyromonas gingivalis* (Jagels et al., 1996; Ramu et al., 2007; Wingrove et al., 1992). It is believed that *M. tuberculosis* is highly adapted to living inside the phagosome of macrophages and so may activate complement to promote uptake into phagocytes. However it has not been studied in detail how it interacts with the complement system nor whether it has evolved means of regulating the extent of complement attack.

The aim of this study was to examine how *M. tuberculosis* activates the different complement pathways and how it interacts with different complement proteins with the primary focus on the interactions between *Mycobacterium bovis* and complement initiators. The genomes of *M. tuberculosis* and *M. bovis* share more than 99.95% sequence identity and *M. tuberculosis* and *M. bovis* BCG share more than 99.9% (Garnier et al., 2003). Therefore, we used *M. bovis* BCG as a model for *M. tuberculosis*. It is known that *M. bovis* BCG can activate the classical and alternative pathways but the mechanism of initiation has not been studied in detail (Ferguson et al., 2004; Ramanathan et al., 1980).

We confirm that *M. bovis* BCG activates complement via the classical and alternative pathways. MBL and L-ficolin binding to the bacterium was demonstrated, indicating lectin pathway activation. C1q binding occurs in the absence of antibody but is enhanced by immunoglobulin. Activation of the classical and alternative pathways resulted in the deposition of C3b on the surface of the bacterial cell. Analysis of culture supernatant and cell wall preparations showed no proteolytic activity capable of activating C3. Further binding assays demonstrated that FH also binds to *M. bovis* BCG, suggesting that the bacterium has a means of moderating complement attack.

2. Materials and methods

2.1. Mycobacterial cultures

Liquid cultures of *M. bovis* BCG (Pasteur strain) were grown in Middlebrook 7H9 liquid medium containing 0.2% (v/v) glycerol, 0.05% (v/v) Tween 80, and 10% (v/v) albumin-dextrose-catalase (ADC BD BBL Prepared Culture Medium: Becton Dickinson and Company, UK). Fresh cultures were inoculated from 1 ml glycerol stock of *M. bovis* BCG to make 100 ml cultures. The 'first passage' was grown for 4–5 days at 37 °C in roller bottles at 2 rpm until the bacteria had reached the exponential growth phase ($OD_{600\,nm}$ = 0.80–1.00). Only the first passages of the strains were used for experimental work.

2.2. SDS-PAGE

SDS-PAGE was done by the method of Laemmli (1970). Sample preparation was described by Fairbanks et al. (1971).

2.3. Preparation of antibody-sensitised sheep erythrocytes (EA) for complement classical pathway assays

EA cells were prepared as described by Borsos and Rapp (1967). Sheep erythrocytes (E) were from sheep blood in Alsevers (TCS Biosciences Ltd., Buckinghamshire, UK) and rabbit antibody was haemolysin (Sigma–Aldrich, Poole, UK, S-1389). EA were stored in 2.5 mM sodium barbital, 71 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% (w/v) glucose, 0.1% (w/v) gelatin, pH 7.4 ($DGVB^{++}$) at 10^9 cells/ml.

2.4. Preparation of neuraminidase-treated sheep erythrocytes (NE) for complement alternative pathway assays

Washed E (12.5 ml) in PBS, 0.5 mM EDTA were suspended in 10 mM sodium phosphate, 140 mM NaCl, 0.1 mM PMSF, pH 5.0–5.1 (incubation buffer) and the concentration of cells was adjusted to 1×10^9 cells/ml. For every 2×10^{10} cells, 5 units of neuraminidase from *Clostridium perfringens* (Sigma–Aldrich, N2876) were added and incubated for 1 h at 37 °C in a shaking incubator. The neuraminidase-treated erythrocytes (NE) were centrifuged (3000 rpm, 10 min) and washed as before with PBS, 0.5 mM EDTA then in 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N,N′-tetraacetic acid (EGTA), 7 mM $MgCl_2$, 2.1 mM sodium barbital, 59 mM NaCl, 2.08% (w/v) glucose, 0.08% (w/v) gelatin, pH 7.4 (DGVB-MgEGTA). NE were resuspended in DGVB-MgEGTA and adjusted to a concentration of 10^9 cells/ml.

2.5. Human serum used in assays

Fresh human blood was collected and allowed to clot for up to 3 h at room temperature. The blood was centrifuged for 25 min at 3000 rpm and the serum was collected and frozen immediately then stored at −80 °C.

2.5.1. Mannose-binding lectin (MBL)-depleted serum

Whole human serum (5 ml) was passed at 4 °C through a 5 ml column of mannose–agarose beads (Sigma–Aldrich) equilibrated in 10 mM HEPES, 140 mM NaCl, 5 mM $CalCl_2$, pH 7.4.

2.5.2. IgG-depleted serum

A Hitrap Protein G column (1 ml) (GE Healthcare) was washed with 0.2 M Glycine–HCl, pH 2.2. The column was equilibrated with PBS, 5 mM EDTA. Whole human serum (1 ml) was loaded onto the column and incubated for 10 min. One volume of PBS, 5 mM EDTA was used to wash out the serum. The depleted serum was dialysed against 10 mM HEPES, 140 mM NaCl and stored at −20 °C.

2.6. Purified proteins

Human C3 was isolated as described by Dodds (1993) and was a gift from Dr AW Dodds (MRC Immunochemistry Unit). C3 was converted to $C3(NH_3)$ and then partially to $iC3(NH_3)$ (used as a standard) as described by Sim et al. (1993). Human factors H and I were isolated as described by Sim et al. (1993) and human C1q was isolated by affinity chromatography on non-immune IgG-Sepharose as described by Reid (1982). Human IgG and IgM were prepared from pooled human plasma (HD Supplies, Aylesbury, UK) as described by Johnstone and Thorpe (1996). BSA was N-acetylated as described by Frederiksen et al. (2005).

2.7. Biotinylation of Factor H and C1q

Factor H or C1q (1–2 mg) was equilibrated in PBS, 0.5 mM EDTA pH 8.5 and incubated with 16.7% (w/w) of biotin N-hydroxysuccinimide ester (Sigma–Aldrich) for 4 h at room temperature. FH was then desalted on PD-10 column (GE Healthcare) in PBS, 0.5 mM EDTA, pH 7.5. Biotinylated anti-H-ficolin and anti-MBL antibodies were prepared as described by Krarup et al. (2004).

2.8. Radioiodination of C3

C3 (50–100 μg in PBS) was radioiodinated with 0.5mCi $Na^{125}I$ (Perkin-Elmer Waltham, MA, USA) using iodogen (0.2 mg) as a catalyst. Radiolabelled C3 was separated from free ^{125}-iodide by desalting on a PD-10 column equilibrated in PBS.