Mechanisms of evasion of complement by *Porphyromonas gingivalis*

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1. ABSTRACT

The complement system is an important host response to invading bacteria. Activation leads to deposition on the bacterial surface of C3b and its' inactivation products and phagocytosis of the opsonised bacteria by host cells. Alternatively the entire complement pathway including terminal components C5b-9 may be activated on the cell surface which gives rise to generation and insertion of the membrane attack complex into the bacterial membrane and cell lysis. Bacterial resistance to complement may be by enzyme digestion of complement components or by the generation or acquisition from the host of surface molecules which allow the organism to adopt host complement control proteins. The involvement of surface polysaccharides can be deduced from the very strong association of resistance with the presence of capsule and extended or modified LPS O-antigens in several species. However, in many cases the mechanism is unclear. The proteases of *Porphyromonas gingivalis* breakdown C3 and C5 and prevent the deposition of C3b on the bacterial cell surface. Greater deposition of both C3b and C5b-9 occurs in protease deficient mutants but mutants do not show loss of resistance to complement mediated lysis. Instead, complement resistance in *P. gingivalis* is associated with the presence on the cell surface of an anionic branched mannan and appears independent of capsule serotype.

2. INTRODUCTION

The complement system is an important part of the immune response to invading organisms, mediating attraction of host phagocytic cells to an area of infection and opsonisation of the invading micro-organisms thereby increasing their uptake. In some circumstances, particularly with Gram negative bacteria, the complement system is able to bring about lysis of the invading organism. This may be accomplished without prior exposure to allow the development of acquired immunity and is therefore thought to be important early in life and to have been established early in evolution. Bacteria have adapted to survive in this environment by destroying components of the complement system, by synthesising protective surface layers, by adopting or synthesising carbohydrates with similar patterns to those of the host, or by adopting regulatory molecules from the host. The most successful in this subversion are also some of the most highly pathogenic bacteria.

It is well recognised that defence of the periodontal tissues from subgingival bacteria and their extracellular products is mediated in part via a plasma exudate, gingival crevicular fluid, derived from the extensive capillary network in these tissues and neutrophil-dominated phagocytic cell recruitment to this region. Hence, the twin functions of complement in opsonisation and direct bacterial killing are
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likely to be important elements of the host defence against periodontal bacteria. The survival of a bacterial species in an inflamed gingival pocket will therefore be dependent upon an ability to subvert or deregulate the complement system. Surprisingly few studies have examined the phenomenon of mechanisms of complement evasion by periodontal bacteria. In this review we examine some of the more well characterised strategies applied by other human pathogenic organisms and compare these to our understanding of how the anaerobic gram negative rod, *Porphyromonas gingivalis*, has successfully solved the problem of maintaining itself in a complement rich environment.

3. THE COMPLEMENT SYSTEM

The complement system is comprised of a series of plasma proteins, activated in a cascade. The central molecule of this system is complement C3 which is present in normal human serum at 0.75-1.75 g/l. Cleavage of this molecule can occur through three pathways but all give rise to two active molecules, C3a and C3b. C3a is a soluble factor which acts as an anaphylotoxin while C3b is deposited onto the cell surface and acts as an opsonin. C3b forms part of the C5 convertase, bringing about the next step in the activation cascade, and can also feed back to activate other molecules of C3, giving amplification of activity. Activation of C5 similarly gives rise to C5a, an anaphylotoxin, and C5b which interacts with C6 and C7 to give a complex which briefly has the capacity to bind to the lipid membrane. When C8 binds to this cell associated complex, one or more molecules of C9 may also become associated to form the terminal complement complex which forms a trans-membrane channel, the membrane attack complex. The membrane attack complex has been shown to be particularly important in killing of Gram-negative bacteria (1). Resistance to serum killing has been identified as a virulence trait in pathogenic bacteria and reviewed recently by Rooijakkers and Strijp (2).

Figure 1. Activation routes of the complement system and means of evasion by human pathogens.
Evasion of complement by P. gingivalis

Table 1. Evasion of complement activity by bacteria

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Enzymes

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Other enzymes

from dissociation by binding of properdin. Association of another C3b molecule to the C3 convertase gives the C5 convertase.

The classical pathway is activated following antibody binding to an epitope on the invading organism and a resultant conformational change of the immunoglobulin molecule. Activation of the pathway is initiated by binding of C1q to antigen-antibody complexes and subsequent binding of the serine proteases C1r and C1s to C1q. C1s cleaves C4 and C2 which together form the C3 convertase, C4b2a, on the cell surface of micro-organisms.

The lectin pathway gains specificity through the lectins mannose binding lectin and ficolin. These are pattern recognition molecules which recognise pathogen-associated molecular patterns shared by a broad class of microorganisms. MBL binds to a range of sugars with 3- and 4- hydroxyl groups in the pyranose ring including mannose, N-acetyl mannosamine, N-acetyl glucosamine, fucose and glucose (3). Three members of the ficolin family have been described in humans of which two are thought to be serum lectins. L-ficolin binds N-acetylglucosamine while H-ficolin binds N-acetyl glucosamine and N-acetyl galactosamine. D-galactose and sialic acid, which are more frequently found in mammalian glycoproteins, are not bound by these lectins. Both ficolins consist of a lectin domain and a collagen-like domain and occur in oligomers (4). When binding of MBL or ficolin takes place the lectin pathway is activated through MBL-associated serine proteases of which three (MASP 1-3) have been described. The exact role of each remains to be clarified but it has been reported that MASP-1 cleaves C3 and C2 while MASP-2 cleaves C4 and C2 (5).

Control of the complement system is vital for the host as bystander damage can occur to host cells if the process is poorly regulated. This is partly achieved by the highly labile nature of some of the complement components eg Factor B and C2 and of the complexes formed on activation. In addition there are regulatory proteins for the complement convertases which all contain a series of short consensus repeating units (SCRs). Factor H and FHL-1 are the main fluid phase regulators of the alternative pathway, acting as co-factors for the serine protease factor I in cleaving C3bBb (6). Factor H is a 150kDa plasma protein composed of 20 individually folded SCRs of approximately 60 amino acids based on a framework of four invariant cysteine residues and highly conserved acidic, proline, asparagine, glycine, tyrosine, phenylalanine and tryptophan residues (7). FHL-1 is a 42kDa protein which is identical with the first seven SCRs of Factor H and includes an extension of four hydrophobic amino acid residues at its C-terminus (8). This SCR is also found in the functionally related proteins, C4bbinding protein (C4BP) and decay accelerating factor (DAF, CD55) and in complement receptors CR1 (CD35) and CR2 (CD21). C4BP plays a similar role as co-factor for factor I in the classical pathway (9) It is a 500-kDa plasma glycoprotein, composed of seven identical alpha chains and
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a unique beta chain linked together by a central core (10). C-terminal parts of both chains contain two cysteine residues each and an amphipathic alpha helix region, which are both required for intracellular polymerization of the molecule. The alpha chains contain eight SCRs and the beta chain contains three (11). DAF accelerates decay of the C3 convertases of both the classical and alternative pathways.

Other inhibitors include C1 inhibitor, a member of the serpin family of protease inhibitors, which inhibits the activity of C1r and C1s. Inhibition of the terminal complement complex is brought about by CD59 which binds to C8, C9 components of the MAC and inhibits formation of the lytic pore.

4. MECHANISMS OF EVASION OF THE COMPLEMENT SYSTEM BY BACTERIA

The complexity of the complement system allows the opportunity for subversion by pathogenic bacteria in several ways (Table 1). To date there is little information pertaining to evasion of this system by periodontal bacteria with the notable exception of P. gingivalis. However it is likely that similar strategies will be employed to those described for other organisms whose survival in the host is dependent upon subversion of this important arm of the innate host defence. The following section describes some of these well recognised themes.

4.1. Proteases

Enzymatic cleavage of host defence proteins has been demonstrated in a number of bacteria. Pseudomonas aeruginosa is able to cleave C1q and C3 by the activity of elastase and alkaline protease enzymes (12). An extracellular protease from an oral strain of Staphylococcus epidermidis can degrade complement protein C5 (13). An endopeptidase of Streptococcus pyogenes cleaves C5a and similar enzymes can be demonstrated in other streptococcal strains of human origin suggesting that inactivation of chemotactic signals is a universal virulence mechanism of streptococci (14, 15). Properdin, a complement regulator which promotes activity by binding and stabilising the C3bBb convertase, is cleaved by a cysteine protease of S. pyogenes, SPE B (16).

These studies demonstrate that complement activity is blocked by bacterial degradation of one or more of the components of the complement cascade. However in some bacteria protease activity can truly be described as subversive since the enzymatic cleavage of the complement components generates biologically active fragments. In P. gingivalis degradation of human serum proteins, including complement factors C3 and C5, was first demonstrated by immunological methods and suggested as a means to explain the high pathogenic potential of the organism by the group of Sundqvist (17). The same group demonstrated P. gingivalis can bring about the generation and degradation of complement C5a (18). Prior to the classification of the Arg- and Lys-gingipains (19) Schenkein suggested that degradation of complement may be dependent on the trypsin-like protease activity of P. gingivalis but that the proteases are not likely to destroy fluid phase complement components at the concentrations present in ginvial crevicular fluid (20, 21). He went on to demonstrate that P. gingivalis W83 failed to accumulate 125I-C3 on the cell surface following opsonization with serum due to cell associated proteolytic activity and deposition was increased following treatment with a cysteine protease inhibitor TLCK (22). The group suggested that P. gingivalis protease activity mimicked that of complement factor D, enabling it to activate C3 in serum without significant C3 accumulation on the cell surface (23). This group confirmed that when protease activity is inhibited greater C3b deposition occurs and in addition deposition of C5b-9 is increased. A similar effect is seen in protease deficient mutants (24). However, although our recent studies confirmed these findings, we were unable to demonstrate any loss of resistance to complement mediated lysis in the presence of protease inhibitors or in mutants deficient in gingipains suggesting that there is an additional mechanism of evasion of protease activity or in mutants deficient in gingipains suggesting that there is an additional mechanism of evasion of serum killing which is independent of protease activity.

Wingrove et al reported that the degradation of C3 and C5 by P. gingivalis might lead to generation of active fragments and were able to demonstrate the generation of a C5α-like fragment which is biologically active for neutrophil activation (25). Discipio et al demonstrated that C5 treated with Rgp, one of the Arg-X specific proteases of P. gingivalis, elicited eosinophil polarisation responses which were greater if the C5 had first been oxidised. Polarisation of eosinophils also occurred when oxidised C5 was treated with Kgp, the lysine specific member of the gingipain protease family, although unoxidised C5 was not affected by this enzyme (26). The results of Schenkein and Discipio suggest that P. gingivalis subverts the complement response rather than simply inactivating it.

In addition to cleavage and activation of components of the complement cascade the proteases of P. gingivalis are also able to bring about cleavage of the human C5α receptor and so prevent neutrophil activation (27). Initially this would appear to counteract any advantage gained by the generation of the C5α-like fragment described by Wingove et al (25). However a second receptor for C5α has subsequently been described, C5L2, (28, 29) and the effect of proteases of P. gingivalis on this receptor has not yet been investigated. Unlike C5α receptor, C5L2 is not coupled to G protein intracellular signalling pathways and an anti-inflammatory function for this receptor has been hypothesised (30). Other bacteria achieve inactivation of the C5α receptor by non-enzymatic means. Recently S. aureus has been shown to produce two exoproteins which are able to block activity. Chemotaxis inhibitory protein (CHIPS) blocks receptors for C5α and formylated peptide on neutrophils with high affinity binding (31, 32). Staphylococcal complement inhibitor (SCIN) binds to C3bBb and C4b2a and so inactivates C3 convertases (33).

Some bacteria are able to subvert host complement control using enzyme mediated methods. For example, enterohemorrhagic E.coli 0157:H7 strains secrete a metalloprotease, StcE, which is able to cleave the C1 esterase inhibitor and localise it on the cell membrane (34, 35, 36).
4.2. Outer membrane proteins

Serum resistance of *Bordetella pertussis* has been attributed to an outer membrane protein BrkA (37). BrkA has a predicted molecular mass of 103 kDa and has two RGD motifs, an outer membrane localization signal, and a common proteolytic cleavage site. BrkA inhibits deposition of C4 on the cell surface (38). In other work Berggard et al suggest that complement resistance is dependent on C4BP binding to a site similar to C4b although sequence homology has not been identified (39). In this study binding was shown to be dependent on two sites on the bacterial surface of which one was contributed by filamentous haemagglutinin. A study by Pishko et al (40) investigated the variation in complement sensitivity in other studies and reported that *B. pertussis* grown in broth culture is sensitive to killing by serum complement in non-immune serum but that resistance is acquired in vivo.

Resistance of *Yersinia enterocolitica* to complement killing was described by China *et al* who showed this was mediated by the outer membrane protein YadA and suggested this was brought about through the binding of Factor H (41). More recently resistance has been shown to be dependent on outer membrane proteins rather than LPS by Biedzka-Sarek *et al*. Resistance to complement killing by *Y. enterocolitica* was shown to be dependent in the long term on YadA and influenced in the short term by another outer membrane protein Ail (42).

The nature of the cell wall of Gram positive bacteria confers resistance to complement killing but in addition some Gram positive bacteria possess outer membrane proteins which resist deposition of C3b and its cleavage products conferring resistance to opsonisation and phagocytosis. Several strains of *Streptococci* have this ability. The M protein of *Streptococcus pyogenes* binds C4BP and Factor H resulting in reduced activation of AP on cell surface (43, 44). Similarly  *Streptococcus pneumoniae* has Factor H binding proteins encoded by the *pspC* locus Pneumococcal surface protein C (PspC) and the factor H-binding inhibitor of complement (Hic) (45). Pneumococcal surface protein A (PspA) is a choline binding protein with sequence homology to PspC which confers reduced C3 activation in vivo and in vitro and inhibits C3 deposition (46).

Many Gram-negative bacteria are susceptible to complement lysis and it is possible to demonstrate strain variation of resistance and hence link resistance to specific attributes. The *E. coli* TraT protein binds the host complement regulator CD59 thus preventing insertion of MAC through the lipid bilayer (47, 48).

4.3. Cell surface polysaccharide

Cell surface polysaccharides of bacteria were originally distinguished serologically and their classification as K or O antigens reflects this. Different O and K serotypes result from variation in sugar composition and linkage specificity in the polysaccharides, as well as their substitution with non-carbohydrate residues. Bacterial lipopolysaccharides (LPS) typically consist of a hydrophobic domain known as lipid A (or endotoxin) which is embedded in the outer membrane, a nonrepeating core oligosaccharide, and a distal polysaccharide or O-antigen. The length of this molecule has been associated with resistance to complement lysis in several bacteria. In a study by Byrd *et al* (49) a strain of *Bordetella parapertussis* and a smooth strain of *Bordetella bronchiseptica* possessed high molecular weight O-side chain and were resistant to complement killing whereas a rough strain of *B. bronchiseptica* lacking this side chain was sensitive. *Salmonella enterica*, serovar typhimurium can decorate its cell surface with long or very long LPS molecules depending on the number of polymers of O sugar repeat-units incorporated (50). The degree of complement activation and susceptibility to phagocytosis in *Salmonella* strains show a direct correlation with the length of these repeat-units. In addition the length and density of the repeat units is important for resistance to the bactericidal activity of complement.

K antigen was originally described as being associated with capsule, and more than 80 distinct K serotypes in *E. coli* result from structural diversity in these polymers (51). To date 6 K-serotypes have been described in *P. gingivalis* which contribute significantly to the virulence of the organism (52, 53) and as K negative strains show variation (54) more classifications may yet be made. In outbreaks of *Vibrio cholerae* in Bangladesh, strains isolated in 2002 were more susceptible to the bactericidal activity of serum compared to strains from 1993 to 1995 and the susceptible strains had less capsular material than the earlier resistant isolates (55). Similarly different capsular serotypes of *Staphylococcus aureus* show difference in virulence in a mouse model (56). Mice challenged with a serotype 5′ (CP5) strain showed a significantly higher level of bacteremia than those challenged with serotype 8′ (CP8) strain. CP5 strains were also more resistant to *in vitro* killing by whole mouse blood. The degree of O-acetylation was similar in purified capsular polysaccharides CP5 and CP8 but CP5 showed greater N-acetylation. Contribution of the capsule to the virulence of *Burkholderia pseudomallei* was studied by Reckseidler-Zenteno *et al* (57). The group constructed a capsule deletion mutant by in-frame deletion of the glycosyltransferase gene *wcbB* which was serum sensitive and in a mouse model it showed less ability to persist in the blood. To define the role of the capsule a double mutant lacking both capsule and O-polysaccharide was constructed and *in vitro* serum killing assays carried out with the addition of purified capsular polysaccharide and O-polysaccharide. Some resistance was obtained by the addition of *B. pseudomallei* capsular polysaccharide to the assay but not by the addition of O-polysaccharide. Furthermore preincubation of the capsular polysaccharide with serum before the addition of bacteria increased the survival of the double mutant in serum to the same level as in heat inactivated serum.

However, in *P. gingivalis* serum resistance appears not to be associated with capsule (24). Mutagenesis at the capsule encoding locus in strain W50 at PG0106-PG0120, leads to the loss of capsule expression on the basis of negative staining and serological analysis. However, the resultant capsule minus mutants are not significantly more
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sensitised to killing than the parent W50. Moreover, naturally capsule minus strains such as 381 are also resistant to the lytic effect of complement.

In Neisseria gonorrhoeae sialylation of lipopolysaccharide (LPS) by host CMP-N-acetylneuraminic acid (CMP-NANA), catalysed by bacterial sialyltransferase, renders gonococci resistant to absorption of complement component C3, to killing by immune serum and complement, to killing by phagocytes and to entry into epithelial cell lines (58). In strains of N. meningitidis, sialic acid incorporation into LPS is endogenous and this organism also possesses strain specific polysaccharide capsule which prevents insertion of the complement membrane attack complex (59). Similarly endogenous sialylation of lipopolysaccharide in Haemophilus influenzae has been shown to contribute to serum resistance when wild-type strains were compared with non-sialylated mutants (60). Ram et al have suggested that the complement control protein factor H acts as a lectin for sialic acid and other polyanions such as heparin and most sulphated glycosaminoglycans (61). Factor H acts as a co-factor for factor I mediated cleavage of C3b so that the enhanced interaction of Factor H with the sialic acid of the cell surface could promote the local inactivation of the complement cascade. Complement resistance brought about by binding of soluble host derived complement control proteins factor H and C4 binding protein onto cell surfaces has been confirmed in several strains, including N. gonorrhoeae (62, 63) and Streptococcus pyogenes (44).

Recent studies from our laboratory have now determined the identity of a cell surface polysaccharide responsible for serum resistance. The study of this polymer was initiated during our investigation of the glycosylation of the Arg-gingipains where it became apparent that the glycans attached to these enzymes were immunologically related to a repeating unit cell surface polymer cross reactive with a monoclonal antibody (MAb 1B5) originally raised against one isoform of RgpA (64). Glycosylation of the Arg-gingipains appears to be important for both enzyme stabilisation (65) and for immune recognition (66) of these proteases by the serum IgG response of periodontal patients. Hence characterisation of the post-translational modifications to the Rgps may offer insights into the mechanism of persistence of this organism in the periodontal tissues. We therefore investigated the identity of the MAb 1B5 reactive cell surface polymer. The polysaccharide (APS: anionic polysaccharide) was purified using a combination of gel-filtration and ion-exchange chromatography and the structure determined by 1 D and 2D NMR and GC-MS analysis. APS consists of a branched mannan structure built up of α-1→2 linked Man backbone with α-1→6 linked Man side-chains with two residues of Man-1-phosphate in phospho-diester linkage at →2)Man in two terminal Man residues in the repeating unit. The polymeric structure of APS was retained following dephosphorylation implying that the phospho-diester is in the side-chains and not in the backbone. Given that MAb 1B5 reacts with all P. gingivalis laboratory strains and isolates examined to date, it appears that APS expression is highly conserved in this species.

Synthesis of APS is dependent upon the expression of genes at the porR locus in the P. gingivalis genome (pg1135-pg1142): deletions at this locus or insertional inactivation of porR (pg1138) lead to loss of reactivity with MAb 1B5, a more fragile cell phenotype and loss of electron dense staining at the cell surface as revealed by electron microscopy. However the composition of the O-antigen of LPS and capsule expression is unaffected by these mutations. We recently reported that loss of APS expression is associated with an exquisite sensitivity to complement mediated lysis in P. gingivalis W50. Whether complement resistance is conferred by the anionic nature of the mannan due to its phosphorylation status or whether the presence of the mannan at the cell surface confers a steric hindrance effect on complement deposition at the outer membrane is yet to be established.

Hence it appears that in the case of P. gingivalis, two distinct mechanisms of complement evasion are in operation. The Arg-gingipains are required to mediate complement degradation, particularly C3 activation, in the fluid phase and this in turn leads to reduced deposition of complement components on the cell surface and presumably reduced opsonophagocytosis. When complement deposition does occur, the presence of the branched mannan at the cell surface protects the cell from complement mediated lysis.

5. PERSPECTIVE

Bacteria have developed a multiplicity of strategies to avoid the chemotactic, opsonic and lytic effects of the complement system and the same organism may have several different strategies to obtain complement resistance. Studies in the literature show there is a long history of examination of enzyme activity against components of the convertases of the classical and alternative pathways and against C5. P. gingivalis Arg- and Lys- gingipains have been shown to be effective mediators of complement subversion. However much less is known about bacterial evasion of the lectin pathway, possibly because that pathway was more recently discovered. There are also several studies described above which demonstrate the adoption of host complement inhibitors to the surface of bacteria mediated by outer membrane proteins as in E.coli or by polysaccharide as in Neisseria spp. and H. influenzae. There is no evidence to date that any similar binding of control proteins occurs in P.gingivalis but this possibility has not been fully investigated.

There are multiple examples in the literature of complement evasion mediated by bacterial polysaccharides. However, the mechanisms by which cell surface polysaccharides afford this protection has not always been established. The role of the phosphorylated branched mannan of P. gingivalis in the evasion of complement and the broader issue of the interaction of this surface polysaccharide with the innate defences of the colonised host are still under investigation in these laboratories.
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Evasion of complement by P. gingivalis


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**Abbreviations:** Mannose binding lectin MBL, MBL-associated serine protease MASP, Short consensus repeating unit SCR, C4 binding protein C4BP, Decay accelerating factor DAF, Chemotaxis inhibitory protein of *Neisseria meningitidis* (CHIP), Staphylococcal complement inhibitor SCIN, Lipopolysaccharide LPS

**Key words:** Innate immunity, complement, complement evasion, complement resistance, *Porphyromonas gingivalis*.  

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