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Protease-dependent mechanisms of complement evasion by bacterial pathogens

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Abstract

The human immune system has evolved a variety of mechanisms for the primary task of neutralizing and eliminating microbial intruders. As the first line of defense, the complement system is responsible for rapid recognition and opsonisation of bacteria, presentation to phagocytes and bacterial cell killing by direct lysis. All successful human pathogens have mechanisms of circumventing the antibacterial activity of the complement system and escaping this stage of the immune response. One of the ways in which pathogens achieve this is the deployment of proteases. Based on the increasing number of recent publications in this area, it appears that proteolytic inactivation of the antibacterial activities of the complement system is a common strategy of avoiding targeting by this arm of host innate immune defense. In this review, we focus on those bacteria that deploy proteases capable of degrading complement system components into non-functional fragments, thus impairing complement-dependent antibacterial activity and facilitating pathogen survival inside the host.

Keywords

complement system; proteases; bacterial resistance; complement inhibitors

Introduction

All multicellular eukaryotic organisms, including human beings, are literally besieged by cohorts of different microorganisms. Fortunately most of them live in peace with the host, or inhabit body surfaces and digestive tracks for homeostatic purposes. From time-to-time, however, hosts are exposed to bacterial pathogens, which may cause the disease if they can colonize the host. In addition, even the most welcome commensals must be restricted in their distribution; and promptly eliminated from body compartments which are maintained as sterile. Therefore, over eons of coexistence with bacteria, multicellular organisms have evolved formidable systems of defense against microbial pathogens, which have reached the highest level of sophistications in vertebrates (Rodríguez et al., 2011). Accordingly, we are protected by two immune systems. These are always present and ready for action against any microbial invaders innate immune system, alongside acquired immunity, driven by antibodies and effector cells specifically developed to attack and destroy certain pathogens. Each of these systems contain many components, forming versatile and interacting lines of

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defense that include the restriction of iron availability (Ganz, 2009), receptors that recognizing microbe-associated molecular patterns (Coll and O'Neill, 2010; Bardoel and Strijp, 2011), bactericidal peptides (Kasetty et al., 2011; Bulet et al., 2004), professional phagocytic cells (Parihar et al., 2010; Scott and Krauss, 2012), coagulation and complement systems (Doolittle, 2011; Loof et al., 2011; Matsushita, 2010; Trouw and Daha, 2011), antigen presenting cells (Nace et al., 2012), antibodies and scores of different lymphocytes. At particular stages of bacterial invasion, when initial lines of defense, such as epithelial barriers, are breached, complement plays an essential role in mobilizing the immune response to an invading pathogen. Complement activation, in conjunction with coagulation system, initiates an inflammatory reaction, which is nothing more than a coordinated assault against microbial invaders (Oikonomopoulou et al., 2012; Hamad et al., 2012). Therefore, the ability to circumvent concerted attack by the immune system, from the point of host invasion, is vital for pathogen survival. As humans and microorganisms have co-evolved, bacteria have developed various strategies to manipulate host immune responses, and possess a wealth of mechanisms to evade host defenses. In this context, it is unsurprising that the complement system, the first line of defense, is a primary target for bacterial virulence factors.

Complement is a tightly and precisely regulated system composed of numerous specific receptors and factors that are activated in a cascade-like manner. This system must achieve rapid and accurate recognition of all non-host surfaces (i.e., bacterial cells) to ensure the timely elimination of pathogens. Due to the multifactorial cascading nature of the system, complement activation provides multiple targets for possible interference by bacterial molecules, including complement inhibitors and proteases. Most successful human pathogens have developed multiple parallel mechanisms of evading the complement system (Rooijackers and van Strij, 2007), one of which is the deployment of bacterial proteases that can efficiently degrade complement components. This degradation results in inactivation of the cascade, down-regulation of the assembly of terminal complement complexes, and an inability to lyse bacterial cells, ultimately ensuring bacterial survival inside the host. In this review, we provide a comprehensive overview of current data on bacterial proteases that target the complement system, with particular emphasis on periodontal pathogens. After a brief introduction to the human complement system, we will discuss the current understanding and examples of the various ways in which pathogenic microorganisms use proteases as a means to evade the complement system.

The complement system

Complement is a major arm of the innate immune defense system and functions primarily to recognize and destroy invading microorganisms. Three distinct complement activation pathways ensure that virtually any non-host surface is recognized as foreign and eliminated. The classical pathway involves binding of the C1 complex (composed of the recognition molecule C1q and 2 proteinases, C1s and C1r) to an invading pathogen, either directly or via immunoglobulins. The lectin pathway recognizes, via mannose-binding lectin (MBL), polysaccharide molecules normally present only on microbial surfaces (Fujita, 2002). Lastly, complement can also be triggered by activation of the alternative pathway, which is in a state of constant low-level spontaneous activity (due to the inherent instability of C3) (Gotze and Muller-Eberhard, 1971). Recent data suggests that there may be other complement activation routes, including extrinsic protease pathways, the C2-bypass pathway (Selander et al., 2006), and properdin-mediated direct convertase assembly on microbial surfaces (Spitzer et al., 2007). All of the activation pathways merge at the level of C3 cleavage, which leads to formation of the active fragments C3a and C3b. C3b opsonises foreign surfaces, which enhances clearance by phagocytosis. Deposition of C3b on microbial surfaces also results in the amplification of complement activation through the formation of surface-bound C3

convertase, and subsequent stimulation of the assembly of C5 convertase, which cleaves C5 into C5a and C5b. C5a together with C3a is a powerful anaphylatoxin capable of attracting phagocytes to the site of infection (Peng et al, 2009). C5b on the other hand binds to the active convertase and initiates the terminal pathway. This leads to the assembly of the terminal pathway components C5, C6, C7, C8 and C9, which form the membrane attack complex (MAC). Assembly and subsequent conformational changes result in the generation of lipophilic, membrane-insertion complexes that form pores in the bacterial cell membrane, ultimately resulting in cell lysis (Muller-Eberhard, 1986). This function, e.g. bactericidal activity of serum, is probably effective only against commensal Gram-negative bacteria, which accidentally enter the blood stream. Of note, host cells are protected from bystander damage following complement activation through the expression of membrane-bound endogenous inhibitors, or through the recruitment of soluble inhibitors of complement (Walport, 2001).

The role of complement in immunity against bacteria, however, expands far beyond the direct and specific killing of microbes. This can be inferred from the fact that, although Gram-positive bacteria are naturally resistant to attack by the MAC complex, Gram-positive pathogens have developed sophisticated mechanism to avoid complement activation. Thus, it is clear that opsonisation facilitating clearance of bacteria by phagocytosis and/or complement-induced inflammatory reactions impose a mortal danger to invading pathogens, which is entirely independent of MAC formation. Examples corroborating this contention are apparent from strategies used by different pathogens to disable complement-dependent antibacterial functions of the immune system, and are discussed in the following sections of this review.

Mechanisms of complement evasion

In order for pathogens to avoid recognition and attack by the immune system, it is essential that they circumvent or subvert the complement system, which is the first line of defense. Pathogens utilize a variety of escape strategies to protect against complement attack as a way to facilitate survival inside the host and effectively increase virulence. Three main strategies of complement evasion by microorganisms are (*i*) the use of bacterially-encoded complement inhibitors; (*ii*) acquisition of host complement inhibitors; and (*iii*) complement neutralization via degradation or inactivation of complement components by proteases (Rooijakkers et al., 2007).

Microbial complement inhibitors

Despite being tightly regulated, the nature of the complement activation cascade nonetheless exposes multiple targets for possible interruption or interference by bacteria. Any bacterially-derived complement inhibitor could potentially disrupt this delicate balance and affect complement function. It is somewhat unexpected, therefore, that only a few direct complement inhibitors have been described to date. A CD59-like protein from *Borrelia burgdorferi* (Pausa et al., 2003), for example, is a membrane-bound protein that binds to both C9 and C8 and inhibits MAC formation. Notably, this bacterial inhibitor binds preferentially to the C8 β subunit, whereas human CD59 targets C8 α . Interestingly, a MAC inhibitor expressed on the surface of *Schistosoma mansoni* has been identified that binds to the C8 β subunit (Parizade et al., 1994). It has also been reported that streptococcal inhibitor of complement (SIC) prevents MAC formation by interfering with C5b-C7 and C5b-C8 complexes (Fernie-King et al., 2001). *Staphylococcus aureus* surface-bound protein A (SpA) is another anti-complement molecule. It recognizes the Fc domain of immunoglobulin G (IgG), which results in the blocking of C1q binding sites, thereby interfering with the classical route of complement activation (Cedergren et al., 1993; Gouda et al., 1992). *S.*

aureus is also armed with staphylococcal complement inhibitors, or SCINs. The function of these small, helical molecules is to stabilize C3 convertase in a non-functional state, thereby blocking all three pathways of complement activation (Rooijackers et al., 2005a). Extracellular fibrinogen binding molecule (Efb) is another staphylococcal protein that plays a role in the *S. aureus* anti-complement response. This 15.6 kDa-secreted protein binds C3d, thereby blocking opsonisation, which is required for the activation of the classical pathway, thus decreasing the rate of phagocytic killing of bacteria (Lee et al., 2004).

Acquisition of host complement inhibitors

Trapping of fluid-phase host regulators of complement activation (RCA) is perhaps the most widely documented bacterial strategy for avoiding the complement response. Expression of microbial surface molecules that bind to complement inhibitors and activate them allows pathogens to inhibit the complement response on the bacterial surface. Recruitment of RCA has significant advantages, namely that RCA are endogenous regulators and therefore poised to carry out their native functions. Additionally, RCA are constitutively produced by the host and, as such, are always available in relatively high concentrations. They are also structurally related, which allows a single pathogen-derived protein to bind multiple host RCA.

Factor H is a 150 kDa plasma protein and a key fluid-phase regulator of the alternative pathway. Together with factor H-like protein (FHL-1), it competes with factor B for binding to C3b. Factor H (FH) and FHL-1 also accelerate the decay of already formed C3 convertase (C3bBb) and act as cofactors for factor I-mediated degradation of C3b (Zipfel et al., 2002). C4 binding protein (C4BP) is another potent fluid-phase regulator and is present in plasma at a concentration of 250 µg/ml. It functions as a cofactor for factor I (FI)-mediated degradation of C4b to C4d and facilitates the decay of C2a from C3 convertase (C4b2a), thus inhibiting the formation of new C3 convertase and inactivating the classical pathway of complement activation (Blom, 2002). Recruitment of these three vital RCA to the microbial surface is a well-characterized mechanism of complement evasion by bacteria (Table 1).

Use of bacterial proteolytic enzymes

Degradation and functional inactivation of complement components by bacterial proteases is a key strategy for attenuating a range of host defense mechanisms, both innate and acquired, that are dependent on complement activation. This proteolytic mechanism of immune system evasion has been reported almost exclusively for bacteria. Interestingly, pathogen-derived proteases target a wide range of substrates, including molecules involved in initiating complement system activation such as C1q and immunoglobulins, as well as C3, a key factor in the amplification of the complement cascade, and even terminal complement components (Ward et al., 1973). In addition to direct degradation, bacterial pathogens also co-opt host proteases to attenuate complement activation. The best-studied example of this is the binding and conversion of plasminogen to reactive plasmin, which results in the degradation of C3 and immunoglobulins on bacterial surfaces.

Periodontal pathogens

Periodontitis is a chronic inflammatory condition with an infective etiology that leads to loss of tooth support (Darby and Curtis, 2001). *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are strongly associated with the disease and are considered the main periodontal pathogens. They constitute the so-called “red complex” species and are characterized by the production of high levels of proteolytic activity (Socransky et al., 1998). In this respect, another periodontal pathogen, *Prevotella intermedia*, resembles these red complex species. A number of studies have shown that proteolytic enzymes encoded by

these pathogens are primary weapons of defense against host innate immunity. As is the case for other pathogenic bacteria, interference with the complement system is a necessity since periodontal pathogens encased in the subgingival biofilm on the tooth surface (dental plaques) are bathed in gingival crevicular fluid, in which concentrations of complement are 70% of that seen in plasma. Although the biofilm structure provides some protection against complement, periodontal pathogen species have evolved formidable mechanisms of complement evasion.

T. denticola is resistant to the bactericidal activity of human serum and a serine protease referred to as dentilisin, have been implicated in complement evasion. Although dentilisin cleaves C3 (Yamazaki et al., 2006), recent data indicate that this function may be a redundant mechanism of complement evasion and that protection is rendered solely by the immobilization of FH and FHL-1 on the bacterial surface by factor H binding protein B (FhbB) (McDowell et al., 2009; McDowell et al., 2011; Miller et al., 2012). Interestingly, binding sensitizes the former complement regulatory protein to proteolytic cleavage by dentilisin. The function of the 50 kDa fragment of FH that is released and retained on the surface of *T. denticola* is unknown.

P. gingivalis strains are highly resistant to complement and employ several different mechanisms of complement evasion (Potempa et al., 2003; Holt and Bramanti, 1991), all of which involve to some extent the proteolytic activity of gingipains. Gingipains are cysteine proteinases of *P. gingivalis* and comprise approximately 85% of the general proteolytic activity of the pathogen. There are three members of the gingipain family, lysine-gingipain (Kgp), which is specific for the lysine-Xaa peptide bond, and the arginine-gingipains (RgpA and RgpB), which are specific for the arginine-Xaa peptide bond. Working in concert, gingipains cleave not only constituents of periodontal tissue, including the basement membrane structural protein collagen, but also protective host proteins such as antibodies (Potempa et al., 2000) and components of the complement system. The gingipains exhibit dual functionality in the targeting and degradation of complement proteins. When bacterial load is low, the enzymes preferentially attack the α -chains of C3 and C5, resulting in the release of C3a and C5a, which are potent anaphylatoxins and neutrophil chemotactic factors. At the same time, gingipains also activate the C1 complex, leading to its deposition on surrounding surfaces. These actions promote a certain level of inflammation, which may ensure nutrient availability for the microorganism and promote colonization. Once bacteria are established in the periodontal pocket and gingipains are present at higher concentrations (Popadiak et al., 2007), the enzymes cleave complement molecules into fragments, resulting in functional inactivation of the cascade. Apart from targeting and cleaving diverse complement components, the adhesive domain(s) of high molecular weight RgpA (HRgpA) serves as a crucial ligand for the endogenous complement system inhibitor C4BP, facilitating immobilization of C4BP on the bacterial surface. This immobilization of C4BP serves as a separate strategy that leads to reduced complement activation on the bacterial surface and enhanced survival of the pathogen inside the host. Thus, HRgpA is the first example of a virulence factor that contributes in two distinct ways to complement evasion by a bacterial pathogen (Potempa et al., 2008). Data also suggest that by disturbing the local balance between endogenous proteases and their inhibitors in the periodontal pocket, gingipains may enhance the effects of host proteases on complement activity (Mahtout et al., 2009) (Table 2).

Interpain A is a secreted cysteine protease (Mallorqui-Fernandez et al., 2007) present in the genomes of the majority of clinical isolates of another putative periodontal pathogen, *P. intermedia*. Inactivation of complement by interpain A is similar to *P. gingivalis* gingipains. Interpain A efficiently degrades the α -chain of C3, the central complement factor, thereby inhibiting all three complement pathways as well as amplification of complement

antibacterial activity. Similar to gingipains, interpain A is also able to activate the C1 complex in serum, causing deposition of C1q on inert and bacterial surfaces. This could be important at early stages of infection when localized inflammation may be beneficial for a pathogen. Interpain A has been shown to act in a synergistic manner with gingipains in degrading complement components (Potempa et al., 2009). As *P. intermedia* and *P. gingivalis* are often present simultaneously at sites of infection and are capable of coaggregating, this cooperativity is most likely exploited under physiological conditions (Kamaguchi et al., 2001).

Another periodontal bacterial protease, *T. forsythia* karilysin, also exhibits synergistic activity with gingipains in the degradation of complement components (Jusko et al., 2012). Karilysin is a metalloprotease capable of degrading elastin, fibrinogen and fibronectin, and has recently been shown to play an important role in modulating the complement system (Karim et al., 2010). Karilysin degrades C4 and C5, as well as MBL and ficolins. Notably, not all complement components were targeted by the enzyme in the presence of whole serum, suggesting a high degree of specificity. Like gingipains and interpain A, karilysin exhibits dual functionality, initially cleaving C4 and C5 α -chains when present at low concentrations, followed by secondary degradation of these molecules into small inactive fragments when enzyme load is high. Given that this strategy of dual functionality is shared among periodontal pathogens, we propose a biphasic model of protease action during bacterial invasion and colonization (Figure 1).

Staphylococcus aureus

S. aureus is a human opportunistic pathogen responsible for a wide range of infections, including pneumonia, endocarditis, sepsis, and toxic shock syndrome (Franklin and Lowy, 1998). Recent studies have shown that *S. aureus* is armed with versatile mechanisms for facilitating bacterial survival inside the host and enhancing virulence. C4BP binding to bacteria has been reported and *S. aureus* is also capable of accumulating FH and factor H related protein-1 (FHR-1) on its surface, thus disguising in complement inhibitors (Haupt et al., 2008). Interestingly, the main ligand for FH, referred to as second immunoglobulin-binding protein (Sbi), exhibits unusual anti-complement activity. Together with staphylococcal protein A (Spa), Sbi has been reported to block C1q binding sites and to bind to and inactivate immunoglobulins, thus blocking the activation of the classical complement pathway (Cedergren et al., 1993; Gouda et al., 1992; Atkins et al., 2008). Furthermore, Sbi reduces the uptake of bacteria by human neutrophils in the presence of serum opsonins and promotes the survival of bacteria in human blood (Smith et al., 2011).

S. aureus also expresses small helical proteins, the SCINs, which block complement activation. These molecules stabilize C3 convertase in a non-functional state, thereby stopping all three pathways of complement activation (Roosjakkers et al., 2005a). While SCINs can bind only to assembled convertases, Efb and Ehp bind C3d and induce a conformational change that inactivates C3, thereby preventing the cleavage of C3 and C5 (Lee et al., 2004; Hammel et al., 2007; Jongerius et al., 2007). *S. aureus* chemotaxis inhibitory protein (CHIPS) and superantigen-like-protein 7 (SSL-7) interfere with complement molecules at later stages of the cascade. Whereas CHIPS antagonizes C5aR, thus impairing neutrophil and monocyte responses (Wright et al., 2007), SSL-7 binds C5 with high affinity, thereby depleting complement and decreasing MAC formation (Langley et al., 2005).

Apart from exploiting designated complement inhibitors, *S. aureus* can also proteolytically inactivate complement by co-opting plasmin or via bacterial aureolysin, a metalloprotease. Staphylokinase is one of only a few recently described molecules that exhibit a more sophisticated mechanism of action in protecting *S. aureus* from complement-mediated

antibacterial activity. Not itself a protease, staphylokinase forms a complex with host-derived staphylococcal surface-bound plasminogen and converts the zymogen into plasmin (Grella and Castellino, 1997). Plasmin targets not only C3 and human IgG, thus impairing immunological responses, but is also capable of clearing the IgG, C3b and iC3b already deposited on the bacterial surface, thereby hampering opsonisation (Barthel et al., 2012). Staphylokinase also binds defensins, annihilating bactericidal properties and further contributing to the attenuation of host antibacterial defences.

Aureolysin, a zinc-dependent metalloprotease and member of the thermolysin family (Banbula et al., 1998), is currently the only *S. aureus* proteolytic enzyme with a well-described effect on complement. Aureolysin specifically degrades C3, thus inhibiting all three complement activation pathways and blocking complement-dependent responses such as neutrophil activation and phagocytosis. Interestingly, in contrast to interpain A, gingipains and the streptococcal cysteine protease streptococcal pyrogenic exotoxin B (SpeB), which completely degrade C3, aureolysin cleaves C3 at one specific site located two residues downstream of the C3 convertase cleavage site. Thus, aureolysin generates active C3b and C3a. However, contrary to C3 convertase, which cleaves C3 in the proximity of the bacterial surface to promote opsonisation, aureolysin, as a secreted protease, cleaves the majority of C3 at sites that are distant from the deposition surface. As such, activated C3b becomes subject to degradation by the host regulators FH and FI, resulting in inhibition of the complement response. Additionally, aureolysin can proteolytically inactivate cathelicidin LL-37 (Sieprawska-Lupa et al., 2004). Thus, this enzyme likely contributes significantly to *S. aureus* resistance to the formidable forces of host innate immunity.

Recent studies have revealed a pivotal role of staphylococcal proteases other than aureolysin in IgG-mediated complement activation. Specific anti-staphylococcal IgG bound to *S. aureus* is efficiently cleaved and shed from the bacterial surface. This process can be prevented by serine- and cysteine-protease inhibitors, but not with inhibitors specific for metalloproteases (Fernandez-Falcon et al., 2011). Inhibition of IgG cleavage resulted in elevated deposition of C3b on the surface of *S. aureus* and an increased rate of bacterial phagocytosis by human neutrophils.

Streptococci

Infection by group A streptococci (GAS, *Streptococcus pyogenes*) in humans causes an array of clinical symptoms ranging from mild upper respiratory tract disorders to severe pneumonia and sepsis. Most commonly, infection with GAS causes acute tonsillitis or pharyngitis followed by impetigo in children. Moreover, GAS may be responsible for streptococcal toxic shock syndrome. GAS is well protected against attack by complement. In addition to proteins on the surface of the bacteria that capture host RCA (Oliver et al., 2008; Pérez-Caballero et al., 2004; Berggård et al., 2001a and Table 1), *S. pyogenes* secretes bacterially-encoded potent inhibitors of complement (Ferne-King et al., 2001). Bacteria also secrete proteases capable of degrading immunoglobulins, and endoglycosidase (EndoS), a highly specific hydrolase for the N-linked glycan in the CH2 domain of IgG. Streptopain (SpeB) and IgG-degrading enzyme of *S. pyogenes* (IdeS) cleave IgG within the hinge region, the latter enzyme exclusively targeting IgG. Interestingly, cleavage of IgG into Fab and Fc fragments by IdeS is enhanced by cystatin C, an endogenous inhibitor of cysteine cathepsins that is related to IdeS (Pawel-Rammingen et al., 2002). This represents an interesting example of a virulence factor that has evolved to exploit a host mechanism to control proteolysis. It is clear that the concerted activity of SpeB, IdeS and EndoS attenuates the classical pathway of complement activation on the surface of GAS, thus providing protection against opsonophagocytosis.

SpeB is also directly involved in the protection of GAS against complement. Through the degradation of C3, SpeB inhibits all three pathways of complement activation at the level of C3 convertase (Terao et al., 2008). In addition, SpeB degrades properdin, a protein essential for the stabilization of the alternative pathway convertases on microbial surfaces (Tsao et al., 2006). In the absence of properdin, convertases are very labile and are rapidly inactivated. In this way, SpeB plays a direct role in inhibiting complement activation via the alternative pathway. The formidable anti-complement activity of SpeB explains why, in stark contrast to the wild-type strain, inoculation of mice with an SpeB knockout strain resulted in an influx of neutrophils into the site of infection, enhanced phagocytosis, and efficient clearance of SpeB-deficient bacteria. These results highlight the pivotal role of SpeB in complement evasion by GAS (Nelson et al., 2011).

ScpA is a multidomain cell-envelope protease from *S. pyogenes* (Kagawa et al., 2009) that inactivates C5a via limited proteolysis of the C-terminus, removing part of the region of C5a that interacts with the C5a receptor (C5aR) on polymorphonuclear leukocytes (PMNs) (Wexler et al., 1985). This results in inhibition of C5a-mediated pro-inflammatory and chemotactic signalling and slows the influx of inflammatory cells and removal of bacteria from the initial site of invasion. The importance of ScpA as a specialized virulence factor is underscored by the narrow specificity of this protease (Cleary et al., 1992). The enzyme cleaves only C5a and has no activity against intact C5 or other proteins. Interestingly, C5a peptidases produced by group B streptococci also exhibit similar activity to ScpA (Chmouryguina et al., 1996; Navarre and Schneewind, 1999; Bohnsack et al., 1991).

Streptococcus pneumoniae has evolved a completely different strategy to evade complement antibacterial activity independent of the capsule. Apart from capturing FH (Lu et al., 2006) and C4BP (Dieudonné-Vatran et al., 2009), *S. pneumoniae* expresses a 53 kDa intracellular protein, pneumolysin (Kancierski and Molby, 1987), which is released from the cell during bacterial autolysis. Pneumolysin activates the classical pathway of complement even at sites distant from the microbe, ultimately leading to the depletion of complement and reduced serum opsonisation activity, thus facilitating bacteria survival (Mitchell et al., 1991).

Serratia marcescens

S. marcescens is an opportunistic pathogen responsible for pulmonary infections, urinary tract disorders and keratitis (Lyerly and Kreger, 1983; Maki et al., 1973). Of the three proteolytic enzymes produced by *S. marcescens*, the 56K protease appears to be the most important for virulence, as it has the broadest spectrum of proteolytic activity (Kamata et al., 1985; Matsumoto et al., 1984). The protease is capable of degrading collagen, fibronectin, IgG and IgA, lysozyme and other immune system molecules. Moreover, it was demonstrated that 56K protease inactivates the haemolytic activity of human complement system. *S. marcescens* proteases can cleave purified C3 and C5. Thus, degradation of these complement factors in human serum may lead to the inhibition of MAC formation and impair the lytic activity of complement. The 56K protease may also interfere with complement function via proteolytic inactivation of C1 inhibitor and proteolytic inactivation of C5a (Oda et al., 1990).

The 56K protease attenuated the chemotactic response of peritoneal exudate PMNs and macrophages in a dose-dependent manner in a mouse model of C5a-induced cell migration. This result was confirmed *in vitro* using purified human C5a. These observations help explain the strong correlation between the intensity of keratitis and virulence of *S. marcescens* strains, as determined solely by the level of 56K protease production. Injection of a virulent strain producing a high level of 56K protease into the corneas of experimental animals elicited low level infiltration of PMNs, accompanied by a significant delay in healing. In contrast, injection of a less virulent strain producing 10-times less 56K protease

resulted in high level infiltration of PMNs and prompt healing of the lesion. These data correlate with the ability of 56K protease to inactivate C5a and confirm the importance of this enzyme as a virulence factor (Molia et al., 1989).

***Pseudomonas* sp**

Pseudomonas aeruginosa is a notorious opportunistic pathogen that causes life-threatening infections in humans with compromised immune systems, including pneumonia and blood stream infections. The bacterium secretes two powerful proteases, elastase (PaE) and alkaline protease (AprA), which degrade human complement system components (Hong and Ghebrehwet, 1992). When incubated with purified C1q, both enzymes exhibited preferential activity for the 28 kDa A-chain and 24 kDa C-chain of C1q, with the C-chain being degraded more efficiently than the A-chain. Moreover, PaE and AprA degrade the key complement component C3. Upon incubation with PaE or AprA, C3 (190 kDa) is cleaved into a 120 kDa fragment, leaving an intact 75 kDa β -chain and two α -chain fragments of 41 kDa and 26 kDa. As with other pathogens described earlier, targeting C3 as the central complement molecule leads to functional impairment of all three pathways of complement activation, ultimately interfering with MAC formation and facilitating bacterial survival. AprA also interferes with the opsonisation of bacteria with C3b, essentially blocking phagocytosis and killing of *P. aeruginosa* by human neutrophils, and can reduce the formation of C5a (Laarman et al., 2012). The latter is accomplished by effective cleavage of C2 by AprA, which circumvents the classical and lectin pathways without affecting the alternative pathway. Degradation of C1q and C3 by both PaE and AprA strongly supports the idea that these proteases, particularly if working in concert, are responsible for the ability of *P. aeruginosa* to evade complement-dependent antibacterial activity. *P. aeruginosa* Tuf also appears to play a role in complement evasion through the activation of plasminogen to plasmin, similar to staphylococcal staphylokinase. Thus, endogenous proteases also appear to contribute to the formidable resources of *P. aeruginosa* aimed at deterring the antibacterial activity of the innate immune system by proteolytic degradation of complement factors (Kunert et al., 2007).

Cronobacter sakazakii

C. sakazakii, formerly *Enterobacter sakazakii*, is a Gram-negative, facultative anaerobic straight rod-shaped bacterium. An opportunistic human pathogen, it is responsible for causing severe meningitis, septicaemia, and necrotizing enterocolitis in infants (Kleiman et al., 1981; Hunter et al., 2008). Despite its low prevalence, this bacterium remains a substantial threat due to its high mortality rate (ranging from 40 up to 100%) (Nazarowec-White and Farber, 1987). Relatively little is known about the virulence factors of *C. sakazakii*. Recently, however, an interesting mechanism of circumventing the complement system was described. The key component of this mechanism is a protein referred to as *Cronobacter* plasminogen activator (Cpa) (Franco et al., 2011). A *cpa*-deficient strain of *C. sakazakii* BAA894 (BAA894 Δ cpa) was 35-times less resistant to the bacterolytic activity of normal human serum (12% serum) compared to the wild-type strain BAA894, indicating a pivotal role of Cpa in deactivating complement. Further investigation revealed that Cpa inactivates complement in a dose-dependent manner via proteolysis of C3 and C4b. Interestingly, when present at low concentrations, Cpa cleaves the α -chain of C3 at the same site as C3 convertase to produce C3a and C3b, the latter of which is subsequently degraded by Cpa into small non-functional fragments. This mechanism may contribute to the impaired C3a-mediated signalling in human serum. Apart its direct effect on complement, Cpa also stimulates the activation of plasminogen and, at the same time, facilitates the inactivation of α_2 -antiplasmin (α_2 -AP). This leads to the formation of fully reactive plasmin, which apart from its other functions, also contributes to the enhanced degradation of complement components. Thus, Cpa appears to serve as a multifunctional protein, using a variety of

strategies to manipulate and downregulate human complement. However, its role *in vivo* has yet to be demonstrated.

Enterococcus faecalis

Enterococci are the third most common nosocomial bacterial pathogens and account for various diseases in humans, including bloodstream, urinary tract, abdomen, endocardium, biliary tract and burn wound infections (Kayaoglu and Ørstavik, 2004). Around 90% of all enterococcal infections are caused by *Enterococcus faecalis*. Although relatively little is known about the pathogenic mechanisms of enterococcal infections, recent research has shed light on the influence of extracellular gelatinase (GelE) in circumventing the complement response, thus facilitating bacterial survival. GelE is a multifunctional matrix metalloprotease capable of hydrolysing fibrinogen, collagen and bioactive peptides (Phipps et al., 1987). The protease plays a significant role in biofilm formation (Mohamed and Murray, 2006), is important for the pathogenesis of periapical inflammation, and is involved in translocation across the human intestine. Recently, it was shown that GelE targets the α -chain of C3 in the presence of FI and FH. Notably, in the absence of FI and FH, GelE modified C3 into a C3b-like molecule (C3b') that retained fD cofactor activity for the proteolysis of fB and was able to form a C3 convertase (C3b'Bb). These results clearly indicate that GelE acts as a soluble C3 convertase that is resistant to proteolysis by FI and FH. GelE proteolytically converts circulating soluble C3 into C3b-like molecules, which are immediately inactivated by water, thereby losing their ability to bind to target surfaces. In this way, GelE inactivates C3, resulting in the consumption of this central complement component and functional inhibition of all three pathways of complement activation (Park et al., 2008). GelE also degrades iC3b already deposited on the bacterial surface, thereby preventing the CR3-mediated interaction of PMNs with *E. faecalis* and inhibiting PMN-mediated bacterial killing. These two functions of GelE may play crucial roles in enhancing *E. faecalis* survival inside the host.

Aeromonas sobria

A. sobria causes bacteraemia in patients with cirrhosis and is considered a causative agent of spontaneous bacterial peritonitis in these patients (Choi et al., 2008). Infrequently, it is also a causative agent in travellers' diarrhea and gastroenteritis (Lim, 2009). *Aeromonas* infections often occur through skin wounds, leading to cellulitis and furunculosis. *Aeromonas sp.* releases a number of virulence factors such as hemolysins, enterotoxins and proteases. Recently, a serine protease from *A. sobria* (ASP) was purified and characterized. The 65 kDa enzyme caused vascular leakage and decreased blood pressure (Imamara et al., 2006). The enzyme also activated prothrombin to α -thrombin, a potent coagulation protease (Nitta et al., 2007). ASP has been shown to release C5a from C5, with the newly generated C5a convincingly identified based on its molecular size and antigenicity (Nitta et al., 2008). In terms of the physiological relevance of these findings, it is important to note that C5a was released by ASP directly in human plasma, despite the presence of various other plasma proteins and protease inhibitors. Furthermore, C5 cleavage away from the microbial surface will consume this complement factor preventing formation of C5 convertase, C5b incorporation into the *A. sobria* cell membrane and assembly of MAC, thus protecting the bacterium from cell lysis.

Elevated levels of fully functional C5a in the presence of intact C5aR may lead to increased neutrophil influx into sites of infection and consequently to increased levels of neutrophil-derived superoxide and elastase secreted in response to LPS (Fittschen et al., 1988). Alternatively, exposure of neutrophils to C5a at non-physiological concentrations could result in neutrophil dysfunction and paralysis of signalling pathways due to decreased C5aR content on the neutrophil surface and/or reduced chemotactic responsiveness to C5aR (Guo

et al., 2003). These results support the idea that C5a generation by ASP may impair the complement system and result in the desensitization of neutrophils, thereby facilitating bacterial escape from the human immune system.

Summary

In contrast to the well-characterized interactions between bacteria and various human complement regulators, relatively little is known about how bacterial proteases interfere with the human complement system (Table 3). The recent increase in studies demonstrating how various pathogens use proteases to circumvent the antibacterial activity of the complement system clearly indicates that proteolytic modification of complement factors is a common strategy. The most commonly-targeted component is C3, the central factor of the complement system. Efficient inactivation of C3 hinders all three pathways of complement activation and disables all complement-dependent functions of the host immune system. In addition to addressing C3, most pathogens use either the same or additional protease(s) to block complement activation at various other levels or interfere with effector molecules such as C5a. Notably, a number of microbes use proteases to convert plasminogen into plasmin, which also disables complement antibacterial functions (Figure 2). Although it is impossible to directly compare the effectiveness of the three different strategies in disabling the complement system, proteolysis unquestionably plays an important role in the process. It is likely not coincidental that many successful pathogens employ all three strategies concurrently to evade the complement system, underscoring the pivotal role of complement in antimicrobial immunity.

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Figure 1.

Biphasic effect of periodontal pathogens-derived proteases on the complement system. *P. gingivalis* gingipains, *P. intermedia* interpain A, and *T. forsythia* karylsin, when present at low concentrations (i.e. early in the colonization process or at a longer distance from the dental plaque) activate the C1 complex leading to deposition of C1q on the bacterial surface. Complement activation may eliminate complement-sensitive bacteria, which could otherwise compete with the pathogens for space and nutrients. At higher concentrations (i.e. in a developed biofilm) proteases suppress the physiological activation of the complement cascade by sequential cleavage of C3, C4, and C5 components of complement. The very efficient initial cleavage releases anaphylatoxins and C4a. At the same time degradation of C3b, C4b, and C5b into non-functional fragments synergistically inhibits bactericidal activity of complement thus protecting complement-sensitive bacteria in their proximity and promoting biofilm development. The locally released anaphylatoxins fuel inflammation, resulting in tissue damage and nutrients generation, as well as they are subjugated for immune evasion paralyzing clearance of *P. gingivalis* by phagocytes (Guo et al., 2003; Huber-Lang et al., 2002; Riedemann et al., 2003). Targets for bacterial proteases are depicted by scissors.

Figure 2.

Activation pathways of the complement system and points of bacterial proteases action. Complement cascade initiates through three major pathways. The classical one activates upon binding of antibodies to their corresponding antigens. It results in activation of serine proteases (C1s and C1r), cleavage of C4 and C2 and consecutive formation of C3 convertase (C4b2b). Binding of MBL to mannose residues on the surface of microorganisms activates the lectin pathway, via activation of mannan-binding lectin serine proteases (MASP), which then cleave C4 and C2, resulting in formation of C3 convertase (C4b2b). The alternative pathway starts due to failure to appropriately regulate constant low-level spontaneous deposition of activated C3 on foreign surfaces. Spontaneously hydrolyzed C3 binds factor B

(FB), leading to formation of the initial alternative pathway C3 convertase (C3bBb). All three pathways converge at a central step, involving activation of C3, leading to the generation of the anaphylatoxin C3a and opsonins C3b and iC3b. In the terminal pathway, C5b initiates the assembly of the C5b–9 membrane-attack complex (MAC), which in turn stimulates microbial cell lysis. Targets for bacterial proteases are depicted by red scissors. C1inh, C1 inhibitor.

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Table 1

Bacterial use of complement regulators, both own and host-derived in evasion of the complement system

Complement evasion strategy	Bacteria	Complement regulator	Further information
Acquiring host-derived complement regulators	<i>Streptococcus pyogenes</i>	FH, FHL-1, C4BP	(Oliver et al., 2008); Emm18 as a ligand for FH and FHL1, Enn18 as a ligand for C4BP (Pérez-Caballero et al., 2004); M protein binds FH, FHL1 and C4BP (Berggård et al., 2001a); Fba and M1 as ligands for FH and FHL1; (Pandiripally et al., 2002)
	<i>Streptococcus pneumoniae</i>	FH, C4BP	Binding of FH localized to N-terminal region of ligand – CbpA (Lu et al., 2006), PsPC as a ligand for C4BP (Dieudonné-Vatran et al., 2009)
	<i>Borrelia burgdoferi</i>	FH, FHL-1, C4BP	OspE as a ligand for FH (Hellwage et al., 2001); (Pietikäinen et al., 2010); 43 kDa receptor for C4BP (Kraiczy et al., 2002)
	<i>Pseudomonas aeruginosa</i>	FH	Tuf as a ligand (Kunert et al., 2007)
	<i>Neisseria gonorrhoeae</i>	FH, C4BP	Porins and type IV pili bind C4BP (Ram et al., 2001); (Blom et al., 2001); Por1A binds FH (Ngampaustadol et al., 2008)
	<i>Neisseria meningitidis</i>	FH, C4BP	C4BP binds PorA (Jarva et al., 2005); 33-kDa protein as a ligand for FH (Schneider et al., 2006)
	<i>Yersinia enterocolitica</i>	FH, C4BP	(Kirjavainen et al., 2008); YadA and Ail as ligands for C4BP (Biedzka-Sarek et al., 2008)
	<i>Borrelia afzelii</i>	FH, FHL-1	CRASP proteins involved in binding (Kraiczy et al., 2002)
	<i>Borrelia hermsii</i>	FH, FHL-1	FhbA as a ligand (Hovis et al., 2004)
	<i>Streptococcus agalactiae</i>	FH	Beta protein as a ligand (Areschoug et al., 2002)
	<i>Bordetella pertussis</i>	C4BP	FHA as a ligand (Berggård et al., 2001b)
	<i>Mycoplasma catarrhalis</i>	C4BP	Ubiquitous surface protein A2 (UspA2) as a ligand for C4BP (Nordström et al., 2004); (Singh et al., 2010)
	<i>Escherichia coli</i>	C4BP, CD59	OmpA as a ligand for C4BP (Prasadarao et al., 2002); (Wooster et al., 2006); (Rautemaa et al., 1998)
	<i>Porphyromonas gingivalis</i>	C4BP	Cysteine protease HRgpA as a major ligand (Potempa et al., 2008)
	<i>Treponema denticola</i>	FH, FHL-1	FhbB as a ligand (McDowell et al., 2005)
	<i>Staphylococcus aureus</i>	FH	Sbi as a ligand (Haupt et al., 2008)
	<i>Haemophilus influenzae</i>	FH, C4BP, vitronectin	(Hallström et al., 2007); protein E and Hsf as ligands for vitronectin (Singh et al., 2011); 32-kDa, 40-kDa proteins bind FH (Hallstrom et al., 2008; Hallstrom et al., 2006)
	<i>Salmonella sp.</i>	FH, C4BP	Protein Rck as a ligand for both inhibitors (Ho et al., 2009); (Ho et al., 2011)
	<i>Borrelia recurrentis</i>	C4BP, FH	HcpA, surface receptor binds FH (Grosskinsky et al., 2009); CihC outer membrane lipoprotein binds C4BP (Grosskinsky et al., 2010)

Complement evasion strategy	Bacteria	Complement regulator	Further information
	<i>Acinetobacter baumannii</i>	FH	FH interacts with three outer membrane proteins (Kim et al., 2009)
	<i>Borrelia spielmanii</i>	FH, FHL-1	CRASP as a ligand (Seling et al., 2010)
	<i>Fusobacterium necrophorum</i>	FH	(Friberg et al., 2008)
	<i>Streptococcus equi</i>	FH	Se 18.9 protein as a ligand (Tiwari et al., 2007)
	<i>Leptospira interrogans</i>	FH	LfhA, membrane protein as a ligand (Verma et al., 2006)
	<i>Bordetella pertussis</i>	C1 esterase inhibitor	Vag8 as a ligand (Marr et al., 2011)
	<i>Helicobacter pylori</i>	CD59	(Rautemaa et al., 2001)
Secreting own, bacteria-derived regulators	<i>B. burgdoferi</i>	C59-like protein	Inhibits MAC formation (Pausa et al., 2003)
	<i>S. pyogenes</i>	SIC	Inhibits MAC formation (Fernie-King et al., 2001)
	<i>M. catarrhalis</i>	UspAs	Binds C3 (Hallström et al., 2011)
	<i>S. aureus</i>	SpA, Sbi	Blocks C1q binding sites (Cedergren et al., 1993; Gouda et al., 1992; Atkins et al., 2008)
		SCIN proteins	Stabilize C3 convertase in non-functional state (Rooijakkers et al., 2005a)
		Efb	Bind C3d, inactivates C3 via conformational change, prevents cleavage of C3 and C5 (Lee et al., 2004); (Hammel et al., 2007); (Jongorius et al., 2007)
		Ehp	
		SSL-7	Binds C5 (Langley et al., 2005)
		SSL-10	Binds IgG (Itoh et al., 2010)
		CHIPS	Antagonizes C5aR (Wright et al., 2007)

Table 2
Complement substrates for proteases from *P. intermedia*, *P. gingivalis*, and *T. forsythia*

Protease	<i>P. intermedia</i>	Interpain A	<i>P. gingivalis</i> HRgpA	<i>P. gingivalis</i> RgpB	<i>P. gingivalis</i> Kgp	<i>T. forsythia</i> karilysin
Complement molecule						
C3	+		+	+	+	+
C4	-	*	+	+	+	+
C5		Not tested	+	+	+	+

– not cleaving

+ cleaving

* Interpain A cleaves purified protein, however C4 is protected from cleavage in human serum

Table 3

Bacterial proteases degrading complement system components.

	Protease	Target molecule	Further information
<i>Prevotella intermedia</i>	Interpain A (InpA)	C3	(Potempa et al., 2009)
<i>Porphyromonas gingivalis</i>	HRgpA, RgpB, Kgp	C3, C4, C5	(Popadiak et al., 2007)
<i>Staphylococcus aureus</i>	Staphylokinase (SAK)	C3, IgG	Indirect, via plasmin activation (Rooijackers et al., 2005b; Jin et al., 2004)
	Aureolysin	C3	(Laarman A.J. et al., 2011)
<i>Streptococcus pyogenes</i>	EndoS	IgG	(Collin and Olsen, 2001a; Collin and Olsen, 2001b)
	SpeB	IgG, C3, properdin	(Collin and Olsen, 2001a; Lukomski et al., 1998; Terao et al., 2008; Tsao et al., 2006)
	ScpA	C5a	(Ji et al., 1996)
Group B streptococci	C5a peptidase	C5a	(Bohnsack et al., 1991)
<i>Serratia marcescens</i>	56k-protease	IgG, C5a, C1inh	(Oda et al., 1990; Molia et al., 1989)
<i>Pseudomonas aeruginosa</i>	PaE, AprA	C1q, C3, C2	(Hong and Ghebrehwet, 1992; Laarman et al., 2012)
	Tuf	C3, IgG	Indirect, via plasmin activation (Kunert et al., 2007)
<i>Cronobacter sakazakii</i>	Cpa	C3, IgG	Indirect, via plasmin activation (Franco et al., 2011)
		C3, C4b	(Franco et al., 2011)
<i>Enterococcus faecalis</i>	GelE	C3, iC3b	(Park et al., 2008)
<i>Aeromonas sobria</i>	ASP	C5	(Nitta et al., 2007; Imamara et al., 2006; Nitta et al., 2008)
<i>Bacillus anthracis</i>	InhA	C3, IgG	Indirect. Accelerates uPA-mediated plasminogen activation (Chung et al., 2006; Chung et al., 2011)
<i>Leptospira interrogans</i>	-	C3, IgG	Indirect, via plasmin activation (Vieira et al., 2011)