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The interaction of bacterial pathogens with platelets

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Preface

In recent years, the frequency of serious cardiovascular infections such as endocarditis has increased, particularly in association with nosocomially-acquired antibiotic-resistant pathogens. Growing evidence suggests a critical role for the interaction of bacteria with human platelets in the pathogenesis of cardiovascular infections. This paper reviews the nature of the interactions between platelets and bacteria and the role this plays in the pathogenesis of endocarditis and other cardiovascular infections.

Introduction

Opportunistic pathogenic bacteria can occasionally gain entry to the human circulatory system resulting in a transient bacteraemia. Serious complications of bacteraemia include life-threatening infective endocarditis (IE)[1], disseminated intravascular coagulation (DIC)[2], immune thrombocytopenia purpura (ITP)[3] and even increased risk of myocardial infarction (MI)[4,5] or stroke[6]. All of these conditions are characterised by abnormal platelet function (see below) possibly mediated by a direct or indirect interaction with the invading pathogen.

Bacteria-platelet interactions are characterised by binding of bacteria to platelets either directly via a surface protein on the bacteria[7] or indirectly via a plasma bridging molecule linking bacterial and platelet surface receptors[8]. Platelet activation is a necessary step in thrombus formation and bacteria can induce activation either by a direct interaction with the platelet[7] or indirectly through bridging molecules[8] or secreted bacterial products[9]. Adhesion and activation can be independent processes mediated by different bacterial components and different platelet receptors. As a result bacteria can have an activating phenotype, an adhesive phenotype, both or neither. Different phenotypes may be specific to different diseases. Thus, bacteria with an adhesive phenotype may be critical for colonisation of a cardiac valve in IE while those with an activating phenotype may be necessary for the enhanced platelet consumption in DIC and thrombocytopenia.

The interaction of bacteria with platelets may result in platelet activation. When this occurs in a localized manner the result is the formation of a thrombus (Figure 1). It is our contention that endocarditis can occur on undamaged valves by small circulating thrombi caused by bacteria in the bloodstream. Also, sterile thrombi that form on damaged heart valves can become infected by circulating bacteria. In both cases

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development of the infected thrombus involving bacterial activation of platelets on the surface of a heart valve can result in heart failure or a septic embolism. If the interaction occurs in a more diffuse manner this can result in consumption of the activated platelets leading to thrombocytopenia or if there is an associated activation of coagulation it can result in disseminated intravascular coagulation.

Platelet activation in the laboratory is commonly studied using light transmission aggregometry[10]. This technique is limited in its sensitivity and to obtain a maximal response bacterial cells are used at a concentration much higher than occurs in bacteraemia. There is no getting away from the fact that aggregation studied in an aggregometer is an artificial situation. However, the process of platelet activation in vivo can occur at very low densities of bacteria and can lead to severe thrombocytopenia due to amplification of the activation process. This can be seen for example with H. pylori-mediated thrombocytopenia where there is no evidence of bacteraemia[3]. Platelet activation causes the release of thromboxane A_2, ADP and serotonin all of which are very potent platelet activators. These lead to localized platelet activation and thrombus formation and if they are released to a sufficient extent cause massive consumption of platelets (thrombocytopenia). This is characteristic of some bacterial infections such as those caused *Helicobacter pylori*[11, 12] and haemolytic uremic syndrome caused by *Escherichia coli*[13]. Thrombocytopenia associated with bacterial infections is thought to occur secondarily to disseminated intravascular coagulation (DIC), a generalised activation of the coagulation cascade during bacteraemia that results in the rapid consumption of platelets[14]. However, thrombocytopenia can occur in the absence of activation of the coagulation cascade[15].

Infective endocarditis can be difficult to treat with antibiotics even if susceptible organisms are involved. This is due poor penetration of the thrombus by some antibiotics [16]. Also bacteria in a thrombus are isolated from the immune system as thrombi are devoid of neutrophils. Platelets are an important part of the host defence because of their ability to secrete potent antimicrobial peptides (see below)[17].

The importance of bacterial-platelet interactions in disease pathogenesis is not yet fully understood. In recent years important investigations have been carried out into the mechanisms employed by different bacterial species for adherence to platelets and for their activation. These studies are providing insights into bacterial pathogenesis and allowing identification of targets for the development of novel therapeutics against vascular infection. In this review we will describe the interactions that occur between bacteria and platelets, the bacterial proteins involved, their platelet receptors and the mediators of these interactions. We will also discuss how bacteria use these interactions to facilitate survival in the harsh environment of the vascular system and how these interactions may be targets for therapy or prophylaxis.

Bacterial infections of the cardiovascular system

The frequency of bacteraemic infections has increased dramatically in recent years, in part due to increased use of interventionary procedures including angioplasty and the deployment of stents, catheters, pacemakers and prostheses[18, 19]. Furthermore, the increase in antibiotic resistance has contributed to the increase in bacterial infections which are refractory to treatment.[20] Bacteraemic infection can lead to serious vascular complications, including life-threatening endocarditis[1], disseminated intravascular

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coagulation)[2] and thrombocytopenia[14]. In addition, there is evidence suggesting a role for infection in conditions such as atherosclerosis[21, 22], myocardial infarction[23] and stroke[6, 24].

A wide range of bacteria can cause infective endocarditis (IE; Table 1)[25]. Despite improvements in health care, there has been a progressive change in risk factors for IE[26]. New at-risk groups include intravenous drug users, elderly people with valve sclerosis, patients with intravascular prostheses, those exposed to nosocomial disease, and haemodialysis patients. *Staphylococcus aureus* has now surpassed viridans streptococci as the most common cause of IE in the developed world[27] and taken together, *S. aureus*, streptococci and enterococci are responsible for more than 80% of cases[1, 26]. Native valve IE is classically associated with congenital heart disease and chronic rheumatic heart disease indicating that prior valvular damage predisposes to infection. Prosthetic valve endocarditis (PVE) occurring shortly after surgery is often caused by *Staphylococcus epidermidis* or *S. aureus*, whereas later PVE is often due to streptococci and Gram-negative bacteria such as *Haemophilus* spp, and *Actinobacillus* spp[26]. HIV-1 positive patients sometimes present with IE caused by unusual organisms including *Bartonella, Salmonella*, and *Listeria*[26]. Periodontal disease, dental procedures and oral hygiene practices may lead to bacteraemia caused by oral commensals and occasionally to serious cardiovascular disease such as IE or myocardial infarction[28].

Nosocomial endocarditis is a rapidly growing category with many patients having no cardiac predisposing factors. The predominant pathogens are staphylococci and enterococci, organisms frequently associated with nosocomial sepsis. Importantly, nosocomial endocarditis has a case fatality rate greater that 50%[1, 26].

Infection has also been shown to play a role in coronary artery disease. Patients who have received antibiotics in the previous year have a reduced risk of MI[29-31] while those who have had a bacteraemia have an increased risk of MI[4]. However, recent studies have shown no decrease in incidence of MI with antibiotic use[32, 33]. While specific pathogens, especially *Helicobacter pylori* and *Chlamydia pneumonia* have been associated with increased risk of MI more recent evidence suggests that pathogen burden is more relevant to an increased risk of MI[22, 23, 34, 35]. In addition, animal studies have shown that exposure to *Porphyromonas gingivalis* is as effective as a high cholesterol diet at inducing atherosclerosis[36-38]. Thus, there is evidence to support a role for infection in cardiovascular and cerebrovascular disease and that the burden of infection may be more important than exposure to a specific agent. However the role infection plays in the pathogenesis of atherosclerosis remains to be elucidated. Platelets play an important role in atherosclerosis[39-41]. Platelet interactions with the atherosclerotic lesion are part of the inflammatory process and lead to progression of the lesion. Plaque rupture results in the formation of a thrombus leading to an acute coronary syndrome (ACS). If this thrombus persists and fully occludes the vessel the result is a myocardial infarction.

Infection may play a role in a number of different stages of atherosclerosis: i) trigger vascular inflammation which eventually leads to atherosclerosis ii) accelerate inflammation of an existing lesion by recruiting leucocytes and platelets to the lesion iii) destabilise an existing lesion iv) enhance thrombus formation on a ruptured lesion.

Biology and function of platelets

Platelets are anucleate derivatives of megakaryocytes and are the smallest and most numerous (normal range: 3 x 10^8 to 4 x 10^8 per ml) corpuscular component of blood[42].

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The typical shape of resting platelets is discoid and upon activation they undergo a shape change to a globular form with pseudopodia. Although they are energetically active, platelets lack nuclei and can perform only limited translation from stable megakaryocyte mRNA[43]. Platelets contain granules for storage of important mediators such as ADP, serotonin, calcium and proteins such as fibrinogen, vitronectin, and von Willebrand factor[44]. Granules also contain platelet microbicidal peptides and proteins. Platelet microbicidal proteins (PMP) and thrombin-inducible PMPs (tPMPs) are present in rabbit platelet extracts and thrombin-induced supernatants[17].

Platelets play a crucial role in thrombus formation. Firstly, single platelets bind to a site of damaged endothelium where the underlying extracellular matrix has been exposed. This involves platelet surface receptors for collagen ($\alpha_2\beta_1$ and GPVI), fibrinogen (GPIIb/IIIa) and von Willebrand factor (GPIb/IX/V) and results in capture of platelets and their activation[45]. Upon platelet activation, the fibrinogen receptor GPIIb/IIIa undergoes a conformational change which allows it to bind soluble fibrinogen[46]. At the same time, cytoplasmic granule contents are released triggering activation of other platelets which are then recruited into the growing thrombus plugging the endothelial damage. Figure 3 shows the intracellular pathways that occur during platelet activation.

The ability to activate platelets has been exploited by some endovascular pathogens to promote colonization and avoidance of the host immune responses. On the other hand, platelets exert anti-bacterial effects through release of microbicidal tPMPs upon activation[47] which act against a broad spectrum of pathogens including some strains of *S. aureus*, viridans group streptococci and *Candida albicans*[17, 48, 49].

tPMPs released from platelets include the classical chemokines (kinocidins) platelet factor 4, CTAP-3, RANTES, fibrinopeptide B and thymosin β-4[50-52]. Strains of *S. aureus* that are susceptible to tPMPs in vitro are less likely to cause endovascular infections in humans and are more rapidly cleared from the bloodstream than bacteria that are resistant[53-55]. In experimental endocarditis *S. aureus* cells that are resistant to tPMPs in vitro are more virulent[56]. Resistance to tPMPs in *S. aureus* is due to a combination of several factors including (i) enhanced membrane fluidity[57] (ii) reduced membrane potential[58, 59] (iii) a high proportion of positively charged groups in the cell membrane and the cell wall due to lysinylation of phosphatidyl glycerol and D-alanylation of teichoic acid[60] and (iv) a plasmid-encoded efflux pump[61].

Evidence exists that platelets are capable of engulfing *S. aureus* cells after activation has occurred[62, 63] possibly avoiding immune defences[63]. It is likely that *S. aureus* can survive within platelets as the bacterium possesses many mechanisms for avoidance of innate immune responses[64, 65]. Clearly, the interaction of bacteria with human platelets may have different outcomes and this may depend on the strain of bacteria which is causing the infection.

Platelet-bacteria interactions

The association of coagulation with infection has been well established in nature. The best characterised example is that of the amebocytes of *Limulus polyphemus* (horseshoe crab). These are the only circulating cell in the crab's blood and, while nucleate, are very similar in morphology to mammalian platelets. Upon exposure to bacteria they aggregate and release their granule contents. Thus, the Limulus amebocytes mediate host defense

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and haemostasis. In mammals the platelet has retained the function of the amebocyte despite the development of dedicated immune cells[66]
Early studies on the interaction of bacteria with platelets focused on the effects of endotoxin on platelet activation[67]. A study in 1959 showed platelets interacting with mycobacteria[68] and a series of studies by Clawson and co-workers during the 1970's examined the response of platelets to whole bacteria[69-74]. However the development of a method to measure platelet aggregation[10] and the availability of commercial platelet aggregometers was to lead to further studies of platelet interactions with bacteria. Platelets have also been shown to respond to exposure to antigen-antibody complex[75] as well as to complement[76] and to secrete bactericidal agents[77] in response to activation, suggesting a role for platelets in the immune response to infection. However, these studies were limited by a paucity of information on platelet function and they provided little information on the platelet proteins involved in the interaction and how the interaction resulted in platelet aggregation. Below we describe some of the most important and best characterised bacteria that are known to interact with platelets.

The interactions of *Staphylococcus aureus* with platelets

Probably the best characterized interaction between bacterial cells and platelets are those involving the major IE pathogen *S. aureus*. While the initial studies focused on secreted mediators of platelet activation such as α-toxin[78] Hawiger and co-workers showed that staphylococci can trigger platelet aggregation involving protein A, IgG and the Fc receptor on platelets[79]. Subsequently, clumping factor was shown to be an important mediator of *S. aureus* adhesion to fibrinogen and to be a virulence factor for endocarditis[80] and this interaction was suggested to be independent of GPIIb/IIIa, the platelet fibrinogen receptor[81]. In fact *S. aureus* has several different surface-associated proteins that are capable of binding to platelets and in some cases of stimulating platelet activation (Table). The proteins typically have signal sequences at their N-termini which direct Sec-dependent secretion through the cytoplasmic membrane and at their C-termini a sorting signal for anchoring the protein to the cell wall[82]. Clumping factor A comprises a ~500 residue fibrinogen binding domain which is projected from the cell surface by an unfolded serine and aspartate dipeptide repeat region (Figure 2). It is the prototype of a family of related proteins with structurally similar N-terminal ligand-binding domains. The fibronectin-binding proteins FnBPA and FnBPB have an N-terminal A domain that has sequence similarity to ClfA and which interacts with the same region of fibrinogen as ClfA. The A domains of the FnBPs are linked to the cell surface by a series of tandemly-repeated unfolded fibronectin-binding determinants[83, 84]. In the case of FnBPA, two molecules of fibronectin can interact with each fibronectin binding region[83].

Early studies showed that *S. aureus* could adhere to platelets in a manner that was enhanced by the presence of fibrinogen[85]. A mutant of *S. aureus* that lacked the ability to bind platelets had a transposon insertion in the *clfA* gene implying that a fibrinogen bridge occurred between ClfA on the bacterial surface and a platelet fibrinogen binding protein, probably the integrin GPIIb/IIIa[86]. It was also suggested that ClfA could bind directly to an as yet unidentified protein of 118 kDa on the platelet surface[87]. By studying strains with one or more mutations affecting surface proteins and by expressing surface proteins separately in a heterologous host (eg. *Lactococcus lactis*), clumping factors ClfA

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and ClfB, fibronectin binding protein A and the serine aspartate repeat protein SdrE were all shown individually to be able to bind platelets and to stimulate platelet aggregation[8].

More recently our groups have analyzed in detail the molecular basis of platelet activation by extending this approach to different host strains and by studying cells in different phases of growth. Firstly, the major determinants of the ability of *S. aureus* to promote rapid activation of platelets differ according to the phase of growth of the culture. With cells from stationary phase, ClfA is the dominant pro-aggregatory surface protein[88] whilst with cells from exponential growth the fibronectin-binding proteins are of major importance[89]. This correlates with regulation of expression of the genes that encode the proteins. The *fnbA* and *fnbB* genes are only transcribed in exponential phase of growth[90] whilst *clfA* is transcribed weakly in exponential phase and is stimulated in a SigB-dependent fashion in stationary phase[91]. By using a regulatable promoter to control expression of surface proteins in the surrogate host *Lactococcus lactis* it was shown that a minimum number of ClfA and FnBPA protein molecules were required on the bacterial surface before activation of platelets could occur[88]. Once the threshold was achieved aggregation occurred rapidly.

By studying a mutant of ClfA that did not bind fibrinogen, by using inhibitors specific for platelet receptors and by analysing the soluble plasma proteins required in a reconstituted plasma-free system with washed platelets it was possible to identify the plasma and platelet components involved[88]. For rapid activation to occur the bacteria must express sufficient ClfA molecules on their surface and bind fibrinogen molecules in plasma. GPIIb/IIIa on resting platelets has a low affinity for fibrinogen but can still bind bacterial-bound fibrinogen. However this is not sufficient to stimulate activation. ClfA-specific immunoglobulin must also be present to acts a bridge between the bacterial surface protein and the platelet immunoglobulin Fc receptor, FcγRIIa[88] (Figure2a).

Similar observations were made with the fibronectin binding protein FnBPA which possesses two different but related mechanisms of engaging and activating resting platelets[89]. The fibrinogen-binding A domain behaves in a similar fashion to ClfA and activates platelets via a fibrinogen bridge to GPIIb/IIIa and an IgG bridge to FcγRIIa. In addition, the fibronectin-binding region BCD can independently activate platelets. This requires fibronectin N-terminal type I domains to bind to the bacterial protein by the tandem β-zipper mechanism[83] and to GPIIb/IIIa via the integrin-binding RGD domain[89]. As with ClfA and the FnBPA A domain, specific antibody (in this case recognizing the neo-epitopes created when FnBPs bind fibronectin[92]) is also required in order to engage FcγRIIa[89] (Figure 2b). FnBPB appears not to be as effective at inducing platelet aggregation[93] although our results contradict this[89].

Soluble fibrin, which is derived from fibrinogen by the actions of thrombin, is more potent than fibrinogen in promoting platelet activation and aggregation by *S. aureus*[85, 94]. This suggests that *S. aureus* may have a high affinity for an existing lesion containing a fibrin clot and may play a role in colonising a damaged valve. If fibrin is generated thrombin must be present, which as a potent platelet activator would lead to thrombus formation. However, it does not explain how *S. aureus* can colonize undamaged valves to cause native valve endocarditis. In fact, *S. aureus* is capable of directly activating platelets independently of thrombin generation. This is supported by our observation that the presence of a thrombin inhibitor only marginally decreased the potency of *S. aureus*-promoted aggregation[89].

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In conclusion, rapid platelet activation mediated by ClfA and FnBPs requires two mechanisms of binding bacteria to resting platelets (i) a fibrinogen or fibronectin bridge to the low activity platelet integrin and (ii) an immunoglobulin bridge to the FcγRIIa receptor. It is likely that clustering of FcγRIIa initiates the signal transduction cascade that causes the subsequent activation and aggregation of platelets (Figure 3). Activation thus depends on the presence of specific antibodies which are present in the sera of most, if not all individuals[95].

Although, ClfA and FnbpA/FnbpB appear to be the dominant surface proteins mediating platelet aggregation in stationary and exponential phase respectively, additional factors including ClfB, SdrE and SpA have also been shown to play a role[8]. SpA had previously been reported to bind platelets through the gC1qR/p33, a platelet complement receptor[96]. However, gC1qR/p33 is an intracellular protein on resting platelets and is only exposed when platelet are activated. SpA binds with high affinity to the A1 domain of von Willebrand factor (vWF), a large multimeric glycoprotein which mediates platelet adhesion at sites of endothelial damage[97, 98]. Recent evidence suggests that SpA plays an important role in mediating interactions with platelets under conditions of high shear[62] (see section, Bacteria-platelet interactions under high shear conditions).

Recently a high molecular mass protein called SraP which is homologous to the GPIb binding protein GspB of *Streptococcus gordonii* (see below) was shown to promote binding of *S. aureus* cells to platelets and to enhance virulence in a rabbit model of endocarditis[99]. SraP did not appear to bind to GPIb and it was not clear if its interaction with platelets stimulated activation.

S. aureus can also activate platelets by a slower mechanism that is independent of GPIIb/IIIa. A non-fibrinogen-binding mutant of ClfA (ClfA-PY) caused slow activation of platelets[88], as did SdrE, a surface protein which was previously shown to activate platelets when expressed in *L. lactis*[8], but which does not bind fibrinogen. ClfA-PY and SdrE-expressing bacteria stimulated activation of platelets after a lag phase lasting between 8 and 20 minutes depending on the plasma donor and the level of protein expression on the bacteria cell surface. In both cases IgG specific for the surface protein was required to engage FcγRIIa and also to provide the stimulus for activation of complement by the classical pathway. Assembled complement components on the bacterial cell surface then bound to complement receptors on the platelet surface. Both complement and antibody are required for activation to occur (Figure 2c). Some strains of *Streptococcus sanguis* can also activate platelets in a complement-dependent fashion[100] (see below). Indeed this mechanism might be widespread among bacterial IE pathogens that do not express potent activating molecules like ClfA or FnBPA. To put it in more general terms, any bacterium should be capable of activating platelets (i) when it expresses a surface protein or proteins at a sufficiently high level and (ii) provided there are antibodies to the proteins present. The latter condition is likely to be met because most IE pathogens are commensals and most individuals have been exposed and have antibodies to the bacterial surface proteins in their sera[95].

Interaction of streptococci with platelets

The viridans group of streptococci are usually commensals of the human oral cavity and may contribute to tooth decay. Very occasionally they gain access to the vascular system and cause infective endocarditis. Until recently *S. sanguis* was the most

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common cause of IE but has now been superceded by *S. aureus*[27]. Studies using animal models of endocarditis have shown that the ability of *S. sanguis* to adhere to and activate platelets correlates with increased severity of disease[101].

Initial studies identified M-protein isolated from Group A streptococci as mediating platelet aggregation in an antibody and complement-dependent manner[102]. Subsequently *S. pyogenes*[103] and *S. sanguis* were shown to directly trigger platelet aggregation[104].

A collagen-like component of *S. sanguis* was implicated in platelet aggregation[105]. This was subsequently identified as platelet-aggregation-associated protein (PAAP)[105, 106]. PAAP is a rhamnose-rich glycoprotein of 115 kDa which contains a collagen-like platelet-interactive domain comprising residues Pro-Gly-Glu-Gln-Gly-Pro-Lys[106]. Gong and colleagues reported that PAAP interacts with platelet membrane proteins of 175 kD and 230 kD to mediate platelet binding and aggregation[107]. However, it has recently been shown that the role of PAAP in platelet aggregation is donor-specific[108].

S. sanguis was found to require IgG interacting with the platelet FcγRIIa receptor to mediate platelet aggregation[109]. However, there is no clear association between IgG levels, FcγRIIa polymorphisms and donor variability[110]. A role for complement and IgG was also proposed[100, 111] and anti-GPIb antibodies were found to be inhibitory[7, 112].

A major problem with these studies is that there is considerable variation in the ability of different *S. sanguis* strains to stimulate platelet aggregation. Kerrigan *et al.* identified three different phenotypes for platelet interactions[7]. Type I strains showed strong adhesion and rapid aggregation of platelets. Type II strains had longer lag times to platelet activation and did not support bacterial adhesion to resting platelets while type III strains did not adhere to or activate platelets[7]. Presumably there is potential for a type IV strain that supports platelet adhesion but does not induce aggregation. In fact the data suggests that the ability to support platelet adhesion is distinct from the ability to induce platelet aggregation and both adhesion and aggregation are likely to be mediated by different bacterial proteins as well as by different platelet receptors.

Platelet activation by type I strains is mediated by a *direct* interaction between the bacterial cell and GPIb, the platelet vWF receptor (Figure 4b). The interaction with GPIb was localised to the N terminal 1-225 residues of GP1bα[7]. The *S. sanguis* protein that interacts with GPIb is the glycoprotein serine-rich protein A (SrpA), a homologue of *Streptococcus gordonii* Hsa and GspB proteins[113]. However, deletion of this protein does not abolish aggregation but it does prolong the lag-time[113], in effect converting a type I strain into a type III strain. For type III strains with a long lag time, activation required specific antibody and complement assembly similar to slow activation promoted by *S. aureus* discussed above[109, 111, 114]. The signalling pathway stimulated by a *S .sanguis* strain that induced slow (~12 min) aggregation was shown to be that illustrated in Figure 3A[115]. After rapid phosphorylation of FcγRIIa to initiate the pathway, the proteins became dephosphorylated (Figure 3b) prior to aggregation and were re-phosphorylated once aggregation occurred.

Despite inhibition of fast *S. sanguis*-induced platelet aggregation by antibodies to FcγRIIa there was no evidence for a direct role for IgG since aggregation occurred in an antibody-free system with only fibrinogen present[7]. However, it must be noted that commercial fibrinogen is contaminated with IgG[88] and there may have been sufficient present to support an antibody-mediated response. Alternatively, since FcγRIIa and GPIb

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co-localize it is possible that the anti-FcγRIIa antibody might sterically block GPIb[116].

Another member of the viridans group of streptococci that causes IE is *S. gordonii*[117]. An early report suggested that *S. gordonii* strains could not stimulate platelet aggregation[118]. However, subsequent studies showed that *S. gordonii* can bind to platelet GPIb via GspB[119, 120] and are capable of stimulating platelet activation[121]. It is now well established that *S. gordonii* can adhere to platelets and this interaction may contribute to the establishment of endovascular vegetations characteristic of IE[121, 122]. GspB is a 286 kDa surface-anchored protein that mediates adherence to platelets by interacting with GPIb. GspB is translocated from the cytoplasm to the cell wall by a dedicated transport system comprising, at least in part, of the SecA2 and SecY2 proteins[121, 122]. Several genes encoding glycosyl transferases lie adjacent to the *gspB*, *secA2* and *secY2* genes suggesting that they are involved in glycosylation of GspB[119, 123, 124]. A homologue of GspB known as Hsa, made by a different strain of *S. gordonii* is nearly identical to GspB at its N terminus suggesting it may have a related function[120] and may have the ability to bind to GPIIb/IIIa as well[125]. Hsa is a 203 kDa sialic acid-binding glycoprotein that mediates binding to platelets and platelet aggregation[121]. Both GspB and Hsa mediate binding to sialic acid residues on the platelet receptor GPIb, suggesting that GPIb is a major receptor for *S. gordonii*-platelet interactions[120]. Hsa binds specifically to the N-linked oligosaccharides on the globular N-terminal domain of GPIbα while GspB binds to oligosaccharides in this region as well as in the membrane proximal mucin core[126]. The primary function of Has/GspB is to support bacterial adhesion to salivary proteins[127, 128] and the ability to interact with GPIb appears to be a novel function. Mutation of Hsa been shown to reduce endocarditis in an animal model[129].

S. mitis has been shown to bind to platelets[130] and mediates adherence through surface proteins PblA and B but does not appear to directly activate platelets[118]. *S. pyogenes* has also been shown to induce platelet aggregation[103] and to contain a collagen-like protein[131] while *S. pneumoniae* induces aggregation in an antibody-dependent manner[132].

S. agalactiae is an important human pathogen that causes pneumonia, sepsis and meningitis in neonates. It also poses a serious threat to immuno-compromised adult patients causing arthritis, urinary tract infections and endocarditis. In a similar manner to *S. aureus* ClfA, a fibrinogen-binding protein made by *S. agalactiae* (FbsA) mediates attachment to and aggregation of platelets in a fibrinogen- and specific IgG-dependent manner[133].

Bacteria-platelet interactions under high shear conditions

Most of the experiments analysing bacteria-platelet interactions have been carried out under static or low shear conditions. However the vascular system, especially arterioles, presents conditions of high shear. It is important that the molecular mechanisms of activation are tested under high shear to simulate better *in vivo* conditions. It is likely that hydrodynamic shear will affect the molecular interactions between platelets and *S. aureus* in the vascular system. Pawar and colleagues demonstrated a key role for SpA in mediating platelet activation at high shear rates[62]. It should be noted that SpA binds to vWF with a K_d in the low nM range[97]. We have recently shown that each of the five SpA domains (A-E) can individually bind to the A1 domain of vWF with similar high

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affinity[98]. A monoclonal antibody directed against vWF partially inhibited platelet activation under these conditions and an antibody specific for the platelet receptor for vWF (GPIb) also partially inhibited Spa-mediated activation suggesting that an SpA-vWF cross-bridge links SpA on the bacterial surface with the platelet vWF receptor GPIb (Figure 4b). It is possible that plasma IgG molecules bound to SpA compete with vWF by occluding the vWF binding site on SpA[62]. However the affinity of the vWF-SpA interaction is sufficiently high to withstand this. The fibrinogen-binding protein, ClfA, was able to mediate platelet adhesion through the GPIIb/IIIa receptor under both low and high shear rates. Hydrodynamic shear affected the receptor specificity of activation-dependent platelet binding to *S. aureus* cells, as evidenced by the transition from a SpA-independent/ClfA-dependent process at low shear to a SpA-dependent process at high shear[62].

When passed over immobilised *S. sanguis* under high shear conditions, platelets first rolled and then adhered, subsequently forming a large thrombus. This is characteristic of the interaction of platelets with von Willebrand factor bound to exposed collagen at damaged endothelial surfaces, a mechanism that occurs during blood clot formation. Although the rolling mediated by vWF only occurred under high shear conditions, platelet rolling over immobilised *S. sanguis* could also occur under low shear conditions. This interaction was shown to be mediated by GPIb on the platelet and by SrpA on the bacteria[113].

Thrombus formation by *Streptococcus pyogenes* has also been studied under conditions of high shear[134]. *S. pyogenes* M protein promoted thrombus formation with fibrinogen- and M protein-specific IgG being essential, indicating that *S. pyogenes* and *S. aureus* share common mechanisms of rapid platelet activation[134].

Interaction of *Porphyromonas gingivalis* with platelets

The oral pathogen *P. gingivalis* has been shown to accelerate atherosclerosis[36-38] and to secrete a platelet activating protease[135]. *P. gingivalis* has been show to directly activate platelets[136]. This depends on expression of a surface adhesin Hgp44 which is processed from haemagglutinin A by surface associated cysteine proteases. Activation requires antibody in serum to bind to antigens on the bacterial cell surface to interact with FcγRIIa on platelets. A role for GPIbα was indicated by monoclonal antibodies recognizing various domains of GPIbα causing partial or complete inhibition of aggregation and by proteolytic cleavage of the N –terminal domain inhibiting activation. However it is not clear if Hgp44 binds directly to GPIbα or if the platelet glycoprotein's involvement is due to its close proximity to FcγRIIa.

Interaction of *Helicobacter pylori* with platelets

Helicobacter pylori causes gastritis, peptic ulcers and gastric carcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and pernicious anaemia[137]. Recent evidence also suggests that *H. pylori* may have pathogenic effects outside of the gastrointestinal tract. Infection with *H. pylori* seems to increase the risks of atherosclerosis[138, 139] immune thrombocytopenic purpura (ITP)[3, 11, 12]. ITP is caused by destruction of platelets, probably by auto-antibodies. *H. pylori* infections have been identified in patients with ITP and platelet counts increased after elimination of infection[3]. Furthermore some *H. pylori* strains could induce platelet activation and

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aggregation which appears to be mediated through initial interaction of von Willebrand factor (vWF) with bacteria in the blood followed by binding to GPIb, the platelet vWF receptor. Function-blocking antibodies specific for either vWF or GPIb disrupted *H. pylori*-mediated platelet aggregation[140]. Specific anti-*H. pylori* antibodies were also required as was an interaction with FcγRIIa. The requirement for vWF in *H. pylori*-induced aggregation suggests a different mechanism compared to *S. sanguis* which also requires GPIb but not vWF[7], although binding of bacterial-specific IgG to the platelet FcγRIIa receptor is common to both bacteria.

Campylobacter fetus infection has been associated with thrombosis in a number of case reports[141] although there is no information on a potential interaction with platelets although *C. rectus* has been shown not to induce platelet activation[142].

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Borrelia-platelet interactions

Borrelia burgdorferi is a tick-borne spirochaete that is the causative agent of Lyme disease, a chronic, multisystemic infection affecting humans in specific geographical regions in the USA and Europe. After several days of localised skin infections at the site of the tick bit, the spirochete disseminates to multiple tissues including the synovium, the central nervous system and the heart[143]. *B. burgdorferi* binds to activated platelets via the GPIIb/IIIa platelet receptor[144]. This interaction may occur through a *B. burgdorferi* surface protein p66[145]. However, it is not clear whether binding occurs directly or through a plasma protein. A prominent feature of Lyme disease is vascular injury. This may result in localised platelet activation and aggregation, which may then lead to *B. burgdorferi* adherence to platelets. This interaction may contribute to colonisation and/or dissemination to other regions of the vascular system.

Borrelia hermsii is a tick-borne spirochaete, which is the causative agent of relapsing fever. *B. hermsii* grows to high density in blood and platelet-spirochete complexes have been observed in blood smears of relapsing fever patients[146]. Similarly to *B. burgdorferi*, *B. hermsii* was shown to bind to platelets through the GPIIb/IIIa receptor of both activated and resting platelets[147]. In contrast, *B. hermsii* induced platelet activation in the absence of added fibrinogen[147]. *B. hermsii* infection of mice resulted in severe thrombocytopenia and spirochete complexes were detected in the blood of infected mice, however, there was no evidence of platelet activation and binding of the spirochaete to the platelets was independent of GPIIb/IIIa[148]. This suggests that the platelet-bacterial complexes are cleared from the circulation and that the activation process in mice platelets may differ from that of human platelets.

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A general mechanism for platelet activation mediated by surface proteins of vascular pathogens.

Increasing evidence indicates that many bacterial pathogens employ a similar basic mechanism for mediating platelet activation. Initial adhesion of bacteria to platelets occurs either through direct adherence or more commonly through a plasma protein bridge with a platelet receptor, usually GPIIb/IIIa or GPIb. This interaction in itself is insufficient for platelet activation but requires circulating antibody specific for the bacterial surface protein to engage the platelet FcγRIIa receptor. This leads to platelet activation and results in platelet aggregation and thrombus formation. Many of the pathogens that cause IE are normal human commensals to which the host has been

exposed so that low levels of specific antibodies are present in blood[95]. Bacteria seem to utilise these antibodies to subvert the immune response to promote pathogenesis. High levels of antibodies will actually prevent activation by blocking the ligand-binding activity of the surface protein. In this case a slower complement-dependent mechanism of platelet activation can be employed by the pathogen. With *Helicobacter pylori*[140] only the sera of individuals who have been infected have antibodies that would be capable of promoting activation.

Bacterial extracellular factors which mediate platelet interactions

The α-toxin of *S. aureus* can potently induce aggregation of platelets and contribute to coagulation of blood[9], even at concentrations below those which cause cytolysis. α-toxin initiates prothrombinase complex formation on platelets[149]. The process resembles that of ionophore agonists by creating ion channels that simulate Ca^{2+} flux (Figure 3C). Paradoxically, elevated expression of α-toxin by *S. aureus* leads to reduced virulence in a rabbit model or endocarditis[150]. This was likely to be due to excessive damage to platelets causing release of PMPs rather than reduced clearance during the bacteraemic phase or to reduced adherence of bacteria to thrombi.

S. aureus can produce at least one protein which blocks activation of platelets. The extracellular fibrinogen binding (Efb) protein is made during post-exponential growth. It binds fibrinogen and can also bind directly to a receptor on activated platelets[151, 152]. It enhances fibrinogen binding to platelets independently of GPIIb/IIIa (Figure 5) but blocks platelet aggregation, presumably by altering the conformation of GPIIb/IIIa-bound fibrinogen so it cannot crosslink with another platelet (Figure 5). An *S. aureus efb* mutant showed decreased virulence in a model of staphylococcal wound infection and the presence of Efb contributed to a delay in wound healing[151, 152]. Enterotoxin B secreted from *S. aureus* has also been shown to inhibit thrombin-induced platelet aggregation[153]. This suggests that activation of platelets by *S. aureus* may be disadvantageous in certain disease situations and that *S. aureus* has evolved mechanisms, via Efb and enterotoxin B, to avoid this outcome. The *S. aureus efb* mutant was not attenuated in an endocarditis model[152].

Bacillus anthracis express two toxins that potently inhibit aggregation of platelets by natural agonists. The edema toxin (ET) is a calmodulin-dependent adenylate cyclase that activates cAMP dependent protein kinase A inside cells and platelets, an event which is likely to initiate events leading to failure to aggregate[154]. The lethal toxin has a completely different mode of action by suppressing p42/44 and p38 mitogen-activated protein kinase pathways[155]. The action of these two toxins suggests that the platelet is an important target in the pathogenesis of anthrax and helps explain the haemorrhages which are a feature of the infection. Haemorrhages also occur during whooping cough caused by *Bordetella pertussis* adenylate cyclase toxin which is attributed to inhibition of platelet function probably by a similar mechanism to *B. anthracis* ET[156].

Periodontitis is a chronic infectious disease in which proteolytic activity contributes to the destruction of tooth-supporting tissue[28, 157]. Several studies have linked periodontal disease to heart disease[28, 157-159]. *Porphyromonas gingivalis* is one of the major causes of adult periodontitis[160]. Gingipains, a family of cysteine proteases produced by *P. gingivalis* contribute to the pathogenesis of periodontitis[161, 162]. Gingipains can directly activate platelets resulting in platelet aggregation which required

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the proteolytic activity of the gingipains and involved stimulation of protease-activated receptors (PAR-1 and PAR-4) on the platelet surface[163]. This mechanism is distinct from activation promoted by surface adhesins on *P. gingivalis* cells[136] described above. *Pseudomonas aeruginosa* occasionally causes infective endocarditis, particularly in intravenous drug abusers[26]. *P. aeruginosa* produces a phospholipase C (PLC) enzyme which stimulates platelet aggregation in a manner which is dependent on the enzymatic activity PLC[164]. Water soluble extracts of H. pylori have been shown to induce platelet aggregation[165]. *S. mitis* has been shown to secrete a platelet activating factor[166] while *S. mutans*-induced platelet aggregation is mediated by a soluble polysaccharide in an antibody-dependent manner[167].

Outlook for novel therapeutics and avenues for future research.

The discovery of a common mechanism of platelet activation by several important pathogens provides increased hope for the development of novel therapeutics for treating serious vascular infections such as IE. In particular, the identification of the crucial role of the FcγRIIa receptor in bacteria-induced platelet activation represents an interesting target for further investigation. Additional analysis will determine if this is a truly universal mechanism. The identification of several host factors that are important for bacterium-platelet interactions suggests that some individuals might be more susceptible to or conversely, more resistant to serious complications of bacteraemia. In addition, several bacterial factors that promote interaction with platelets and contribute to pathogenesis have now been identified. Development of specific monoclonal antibody therapies against these proteins may prove to be useful in treating or preventing serious cardiovascular infections. Ideally small molecule inhibitors of the interaction between platelets and bacteria could be developed which would have the advantage of by-passing antibiotic resistance mechanisms. Accordingly, they would be ideal for prophylaxis of infection in susceptible individuals and would allow restriction of antibiotic therapy to cases of overt infection.

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Table. 1 Bacterial agents of vascular infections and reported interactions with platelets

Bacterial pathogen	Vascular disease association	Platelet adherence/ aggregation	Bacterial factors	Host factors
Staphylococcus aureus	IE	+[164]	ClfA[8, 88]	Fibrinogen GpIIbIIIa FcγRIIa IgG[88, 89]
			ClfB[8]	" "
			FnbA, FnbB[89]	Fibrinogen fibronectin GpIIbIIIa FcγRIIa IgG[88, 89]
			SpA[8, 62]	vWF
			SdrE[8]	complement IgG
			SraP[99] Efb[152]	Not known
			α-toxin[9]	Not known[88, 89]
Staphylococcus epidermidis	IE	+[168]	SdrG (unpublished data)	Fibrinogen GPIIb/IIIa FcγRIIA IgG
Staphylococcus capitis	IE	+[168]	?	?
Streptococcus sanguis	IE	+[118]	SrpA PAAP[113]	GP1b FcγRIIa[113]
Streptococcus agalactiae	IE	+[133]	FbsA[133]	Fibrinogen IgG[133]
Streptococcus pyogenes	IE	+[103]	M protein	FcγRIIa
Streptococcus gordonii	IE	+[122]	GspB[119, 120] HsA[121]	GP1b[119, 120]
Streptococcus pneumoniae	IE	+[169]	?	?
Streptococcus mitis	IE	+[130]	PblA and B, Sm-hPAF	?
Enterococcus spp	IE	+[170]	?	?

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Neisseria gonnorrhoeae	IE	?	?	?
Pseudomonas aeruginosa	IE	+[164]	PLC [164]	?
Porphyromonas gingivalis	IE	+[163]	Gingipains[171] Hgp44	FcγRIIa[171] GPIbα?
Helicobacter pylori	Myocardial infarction	+[140]	?	vWF GP1b IgG[140]
Borrelia burgdorferi	Lyme disease	+[144]	p66[145]	GPIIb/IIIa[144]
Borrelia hermsii	thrombocytopenia	+[147]	?	GPIIb/IIIa[147]
Haemophilus influenzae	Myocardial infarction	?	?	?
Actinobacillus	IE	+[172]	?	?
Candida spp.	IE	+[173]	?	?
Chlamydia pneumoniae	atherosclerosis	+[174]	?	?
Listeria monocytogenes	IE	+[175]	?	?
Staphylococcus aureus	Delayed wound healing	Inhibition	Efb	?
Bacillus anthracis	Haemorrhage	Inhibition	Oedema toxin Lethal factor	Protein kinase A MAP kinase pathway

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Figure 1. Platelet activation and aggregation.
Sequence of events that occur when bacterial cells bind to platelets and stimulate activation. A and B: Bacteria bind to a platelet receptor, either directly or via (a) bridging ligand(s). C. Signal transduction cascade is stimulated. This leads to activation of the platelet integrin GPIIb/IIIa and expression of more molecules on the platelet surface. D. The activated GPIIb/IIIa binds to soluble fibrinogen in plasma which causes aggregation.

Figure 2. Mechanism of bacteria-mediated platelet adherence and activation. Diagrams summarizing platelet activation involving the immunoglobulin receptor FcγRIIa. A. Rapid activation involving a fibrinogen bridge between a bacterial binding protein (ClfA, FnBP) and low affinity resting GPIIb/IIIa and specific IgG which engages FcγRIIa. B. Rapid activation involving a fibronectin bridge between FnBP and the platelet integrin GPIIb/IIIa and IgG specific for neo-epitopes created when FnBP binds fibronectin which engages FcγRIIa. C. Slow activation involving antibody recognizing a surface protein which stimulates complement fixation. Activation involves IgG engaging FcgRIIa and complement being recognized by a platelet complement receptor

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Figure 3. Signalling Pathways in Platelet Activation[176-178]

A: Platelets contain a low affinity receptor for the Fc portion of IgG called FcγRIIa. Upon binding the Fc region of antibody the receptor dimerizes. This requires antibody to be bound to a surface or to be agglutinated. Not only can Fc form homodimers but it can also form heterodimers with GPIb, the von Willebrand factor receptor or complement receptors. After dimerization tyrosine residues in the ITAM (immunoreceptor tyrosine-based activation motif) domain are phosphorylated by a Src kinase, probably Lyn. The tyrosine kinase Syk then binds to the phosphotyrosine residues and phosphorylates tyrosine residues in the adapter protein LAT. This results in the recruitment and activation of $PLC_{\gamma 2}$. $PLC_{\gamma 2}$ produces inositol triphosphate (IP3) which binds to a calcium ion channel allowing an influx of calcium from internal stores. Also produced is diacylglycerol (DAG) which activates protein kinase C (PKC) which triggers downstream events ion activation. Phosphoinositide-3 (PI3) kinase binds to dimerized receptor and plays an important role in actin assembly which is essential for platelet aggregation.

B. The signal is terminated by the action of a number of phosphatases: PTP1B acts to dephosphorylate LAT, SHP-1 is recruited to the phosphorylated receptor and acts to dephosphorylate Syk while SHIP-1 is an inositol phosphatase and acts to inhibit PI3 kinase activity.

C: Platelets can be activated by many different agents including ADP, thrombin, collagen, and adrenaline. Activation of receptors by these agonists initiates two distinct signalling pathways. The first is the mobilization of phospholipase A_2 which cleaves arachidonic acid (AA) from the membrane of the platelet. This arachidonic acid is then converted to prostaglandin H_2 (PGH_2) by cyclooxygenase (COX). PGH_2 is then converted to thromboxane A_2 (TxA_2) by thromboxane synthase (TXS). TxA2 is secreted

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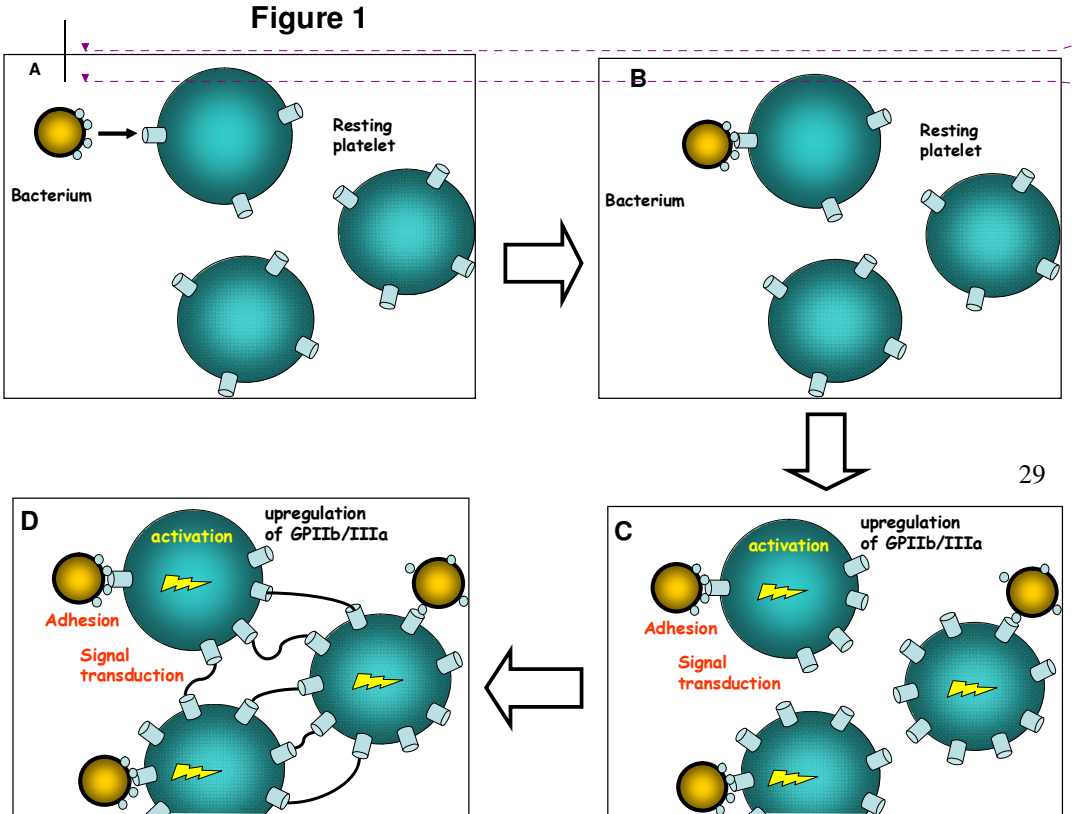
by the platelet and can activate other platelets as well as being a potent vasoconstrictor. A second pathway of platelet activation involves the activation of phospholipase C (PLC) which releases inositol triphosphate (IP_3) and diacylglycerol (DAG) from the platelet membrane. IP_3 binds to its receptor on the endoplasmic reticulum triggering the release of Ca^{2+} from internal stores. DAG acts to activate protein kinase C (PKC). The actions of Ca^{2+} influx and PKC lead to activation of GPIIb/IIIa which can then bind its ligand fibrinogen. As a divalent molecule, fibrinogen can bind to two GPIIb/IIIa molecules simultaneously. If these are on different platelets the result is the formation of a platelet aggregate. Weak agonists only trigger PLA_2 activation while strong agonists trigger PLC activation. In many cases PLA_2 is involved with low doses of agonist and PLC becomes involved at higher doses. Aside from causing the formation of a thrombus, platelet activation causes a number of other responses. Intracellular granules fuse with the membrane and release their contents. These include many vasoactive substances and growth factors as well as microbicidal peptides. Activated platelets also undergo the membrane "flip-flop" where intracellular phospholipids are translocated to the surface. These phospholipids allow the assembly of complexes of coagulation factors including the FXase complex and prothrombinase complex. Thus, activated platelets play an important trigger of the coagulation system. Bacteria that cause activation and aggregation stimulate these pathways by interaction with FcγRIIa or GPIbα.

Figure 4. Bacterial interactions involving platelet glycoprotein GPIb
Binding to GPIb on platelets. A. A model for the interaction of *S.aureus* with platelets involving a von Willebrand factor bridge between protein A and GPIb. B. Direct binding to GPIb mediated by streptococcal surface proteins

Figure 5. Platelet interactions of the staphylococcal secreted proteins, Efb and α-toxin. The secreted protein Efb can bind to GPIIb/IIIa and fibrinogen resulting in both enhanced binding to fibrinogen (**A**) and inhibition of platelet aggregation (**B**). α-toxin stimulates platelet activation through prothrombinase complex formation leading to ion channels which stimulate Ca^{2+} flux (**C**).



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ABOUT THE AUTHORS

Professor Tim Foster has a personal chair in Molecular Microbiology in Trinity College Dublin (Ireland). In the early part of his career he studied mechanisms of resistance to antibiotics and heavy metals in bacteria, and performed some of the early work on characterization of transposon Tn*10*. In the mid-1980s, his attention turned to studying molecular pathogenesis *of Staphylococcus aureus*. Successful genetic manipulation of *S. aureus* enabled characterization of many virulence factors. Current research focuses on surface proteins involved in interactions with platelets and in promoting colonization of the desquamated epithelium. He discovered and characterized the fibrinogen-binding surface protein clumping factor A, which is being developed by Inhibitex Inc. as a candidate antigen for vaccines to combat nosocomial infection.

Dr. Dermot Cox has almost 20 years experience studying platelet function. He started in the pharmaceutical industry leading a drug discovery team in the area of novel anti-platelet agents. 10 years ago he developed an interest in the role of platelets in infection. As a platelet biologist his primary interest is in the response of the platelet to exposure to pathogens. He is currently a lecturer in pharmacology in Molecular Cellular Therapeutics in the School of Pharmacy of the Royal College of Surgeons in Ireland.

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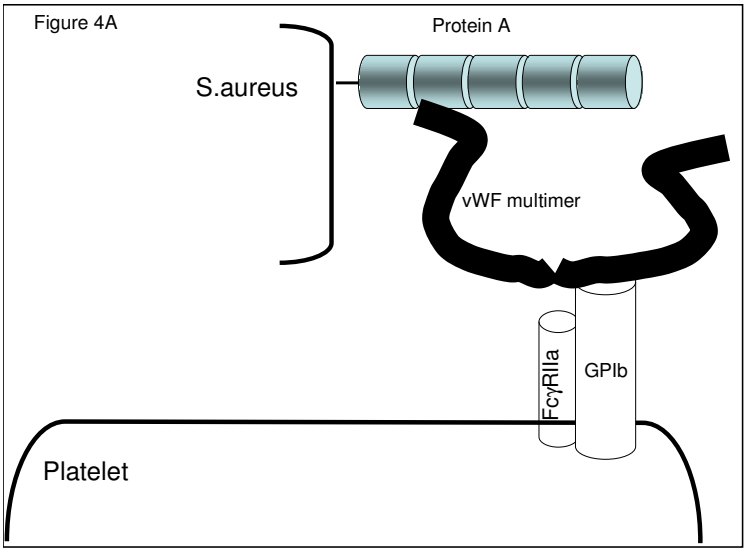


Figure 4A
Protein A
S.aureus
vWF multimer
FcγRIIa
GPIb
Platelet

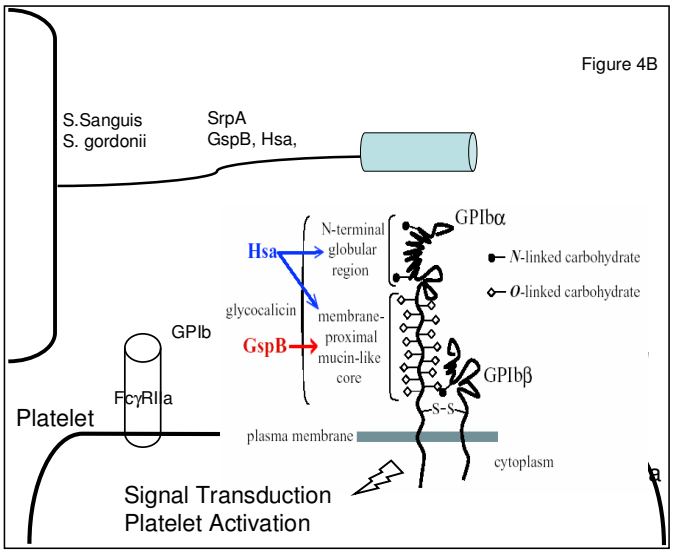
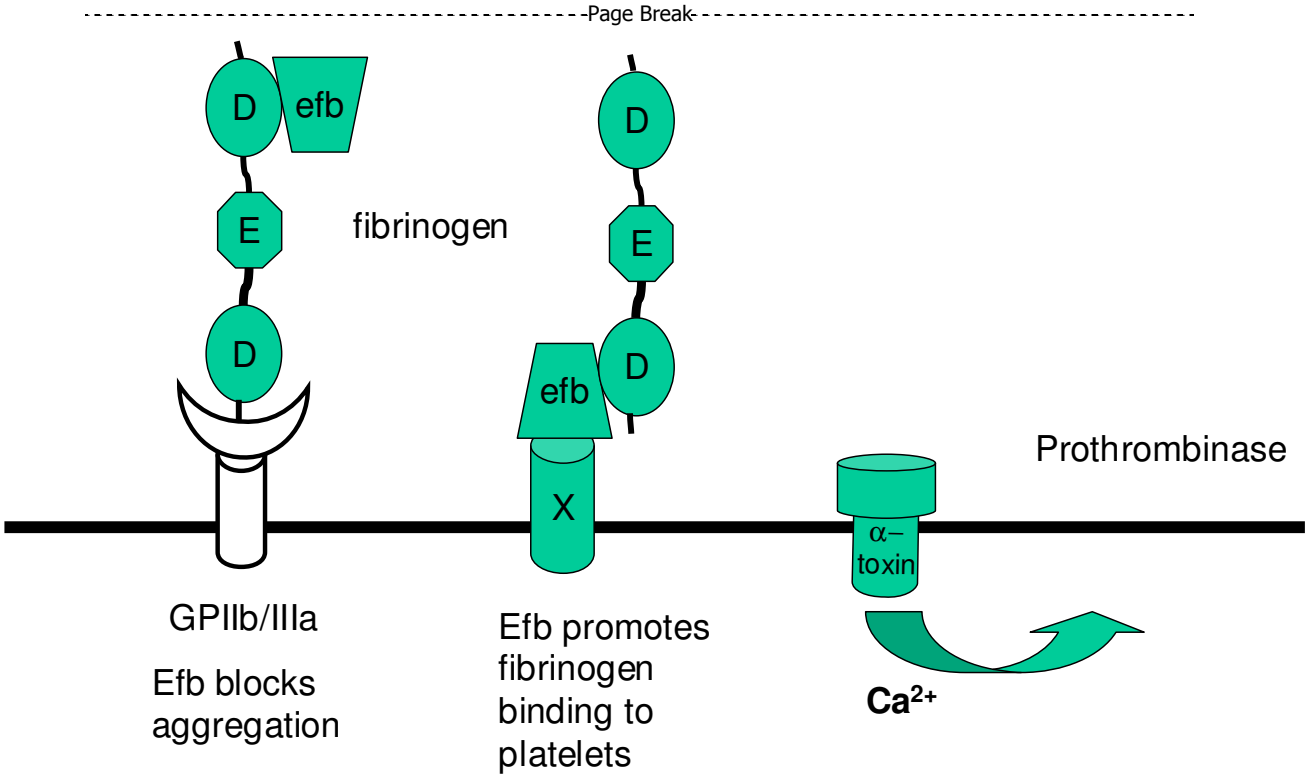


Figure 4B
S.Sanguis
S. gordonii
SrpA
GspB, Hsa,
GPIbα
N-terminal
globular
region
Hsa
N-linked carbohydrate
O-linked carbohydrate
glycocalicin
membrane-
proximal
mucin-like
core
GspB
GPIb
GPIbβ
FcγRIIa
Platelet
plasma membrane
cytoplasm
Signal Transduction
Platelet Activation

Figure 5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Efb promotes
fibrinogen
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platelets
Ca2+