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Biological effects of Extracellular fibrinogen binding protein (Efb) in *Staphylococcus aureus* infection

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Trí caindle forosnat gach ndorcha:
fír, aicned, ecna.

Three candles that illumine every darkness;
truth, knowledge and the ways of nature.

Triads of Ireland

For my family

ABSTRACT

Staphylococcus aureus is a leading cause of human and animal infection. The increasing incidence of antibiotic resistance among strains has complicated treatment of these infections. In order to develop new treatment strategies, it is important to identify and characterise bacterial factors that contribute to infection. *S. aureus* produces a diverse array of virulence factors, among these Extracellular fibrinogen binding protein, Efb. Efb is one of several fibrinogen-binding proteins produced by *S. aureus*. Efb is produced *in vivo* during infection and contributes to the pathogenesis of severe *S. aureus* wound infection in an animal model. This thesis has been focussed on characterising the biological function of Efb in *S. aureus* infection.

We have shown that Efb interacts with the Aα chain of fibrinogen, at a site of functional importance for the interaction between fibrinogen and platelets. We have studied the effect of Efb on platelet function *in vitro* and *in vivo*. Efb bound specifically to a receptor on activated platelets, however fibrinogen was not involved in mediating this binding. Efb stimulated a novel type of fibrinogen binding to the platelet, which did not involve the normal platelet fibrinogen receptor, GPIIb/IIIa. We propose that fibrinogen can also bind to the platelet via Efb and this generates the novel fibrinogen binding mediated by Efb. In the presence of Efb, platelet activation was diminished and Efb also inhibited platelet aggregation in response to various platelet agonists. This antiplatelet effect of Efb was confirmed *in vivo*. Intravenous Efb significantly prolonged bleeding time but had no effect on the coagulation system. This confirms that Efb specifically inhibits platelet function. Moreover, in a mouse model, intravenous Efb rescued 100% of animals from death due to acute thrombosis. This reflects the inability of the platelets to aggregate in the presence of Efb, an effect so powerful that Efb can counteract the massive thrombosis generated in this model.

We have also studied the immune response against Efb and characterised a protective antibody response. Hyperimmune IgG against Efb neutralised Efb and blocked the various biological effects of Efb *in vitro*. IgG against Efb blocked Efb binding to fibrinogen, neutralised Efb and returned platelet aggregation in the presence of Efb to normal. Furthermore, immunization with Efb protected against the development of severe infection in an animal model of *S. aureus* infection. The animals in the vaccinated group developed high titre, specific antibodies against Efb and had significantly less severe infection than those in the unvaccinated group. This establishes Efb as a worthy vaccine candidate for *S. aureus* infection.

Collectively, in this thesis we have elucidated the biological function of Efb and confirmed its importance in *S. aureus* wound infection. Efb is a powerful antiplatelet agent, which impairs haemostasis and wound healing. Efb also represents a worthwhile vaccine candidate for certain *S. aureus* infections.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Marco Palma, **Oonagh Shannon**, Hernan Concha-Quezada, Anders Berg, and Jan-Ingmar Flock. Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* blocks platelet aggregation due to its binding to the α chain. The Journal of Biological Chemistry. 276 (34): 31691-31697. 2001.

II. **Oonagh Shannon** and Jan-Ingmar Flock. Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* binds to platelets and inhibits platelet aggregation. Thrombosis and Haemostasis. 91: 779-89. 2004.

III. **Oonagh Shannon**, Andreas Uekotter and Jan-Ingmar Flock. Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* as an antiplatelet agent *in vivo*. Thrombosis and Haemostasis. Paper in press. 2005.

IV. **Oonagh Shannon**, Andreas Uekotter and Jan-Ingmar Flock. The neutralising effects of hyperimmune antibodies against Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus*. Manuscript. 2005.

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LIST OF ABBREVIATIONS

Efb	Extracellular fibrinogen binding protein
MRSA	Methicillin resistant *Staphylococcus aureus*
LFA-1	Lymphocyte function associated molecule 1
ICAM-1	Intercellular adhesion molecule
MHC	Major histocompatibility complex
ECMBP	Extracellular matrix binding protein
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
Fg	Fibrinogen
Fn	Fibronectin
Cn	Collagen
Pt	Prothrombin
FgBP	Fibrinogen binding protein
ClfA	Clumping factor A
FnBP	Fibronectin binding protein
Eap	Extracellular adherence protein
Emp	Extracellular matrix protein binding protein
IsdA	Iron responsive surface determinant A
Vn	Vitronectin
vWF	von Willebrand factor
ADP	Adenosine di phosphate
TXA_2	Thromboxane A_2
GPIIb/IIIa	Glycoprotein IIb/IIIa
PDGF	Platelet derived growth factor
TGF	Transforming growth factor

PMP	Platelet microbicidal peptides
IE	Infective endocarditis
PBS	Phosphate buffered saline
FPLC	Fast protein liquid chromatography
NaCl	Sodium Chloride
GST	Glutathione *S*-transferase
GSTRR	The N-terminal repeat regions of Efb fused to GST
Efb210	The C-terminal region of Efb fused to six histidines
PPP	Platelet poor plasma
PRP	Platelet rich plasma
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
ELISA	Enzyme linked immunosorbent assay
IgG	Immunoglobulin

1. INTRODUCTION

Staphylococcus aureus

During the early 1880s, Alexander Ogston convincingly showed that a bacterium was the cause of certain abscesses in humans. In recognition of its appearance microscopically he called it Staphylococcus, derived from the Greek staphyle (bunch of grapes) and kokkus (berry). *Staphylococcus* now describes a genus that includes both human pathogens and non-pathogenic staphylococci. *Staphylococcus aureus* is a significant cause of both human and animal infection, causing both community-acquired and hospital-acquired infections. In fact, *S. aureus* is one of the leading overall causes of nosocomial infection (88). *S. aureus* has a broad tropism for human tissues, with an ability to cause infection in the majority of tissues and organs. A list of the various infections for which *S. aureus* is a significant etiological agent is included in Table 1.1.

Table 1.1 Infections or syndromes for which *Staphylococcus aureus* is a prominent pathogen.

Soft tissue infections
▪ Furuncle or carbuncle
▪ Impetigo bullosa
▪ Abscesses
▪ Cellulites
▪ Surgical wound infections
Invasive infections
▪ Bacteraemia
▪ Endocarditis
▪ Septic arthritis
▪ Septic shock
▪ Osteomyelitis
▪ Hospital acquired pneumonia
Food borne gastroenteritis

Prior to the discovery of antibiotics, a *S. aureus* infection was tantamount to a death sentence and mortality rates were as high as 80%. With the discovery of penicillin and the advent of the antibiotic era, *S. aureus* was heralded as a defeated adversary. However, antibiotic resistant strains emerged rapidly and *S. aureus* was firmly re-established as an infectious force to be reckoned with. Antibiotic resistant strains of *S. aureus* are rapidly becoming the norm and continue to complicate treatment options in the modern clinic. The worldwide incidence of Methicillin-resistant *S. aureus* (MRSA) is steadily increasing, and these strains frequently carry resistance genes to other antibiotics. The incidence of MRSA ranges considerably, from 1-5% in Northern Europe, 45% in the United Kingdom and reported levels of 60% in the United States. MRSA infections are associated with increased mortality rates and prolonged hospital stays for the patient and treatment options are usually limited. Initially, MRSA was considered to be associated with the hospital environment but in recent years MRSA infections have been encountered in the community (CA-MRSA). Vancomycin is often the only remaining antibiotic that is effective against MRSA infections, however reports of strains with reduced sensitivity to vancomycin have been increasing. In 2002 the first confirmed strains of vancomycin resistant *S. aureus* were separately identified in the USA (20) (154). The search for new antibiotic targets is intense, renewing interest in the field of staphylococcal pathogenesis.

S. aureus is a common commensal of humans and can be isolated from many skin sites. The primary ecological niche is in the anterior nares and elimination from the nose results in subsequent elimination from other sites (103). Carriage patterns vary between individuals and three categories have been defined. In a healthy adult population about 20% of individuals are persistent carriers, about 60% are intermittent carriers and about 20% are non-carriers (111). Host factors, environmental factors (76) and bacterial factors (105) (117) (151) all contribute to the carrier status, however the exact mechanisms involved remain to be elucidated. A link between nasal carriage and the development of staphylococcal infection has been established (19) and many infections are of endogenous origin (144) (152).

The immune response against *S. aureus*

Mucous membranes constitute the body's first line of defence against bacteria. The aim of the mucosal membrane is to provide a physical barrier to *S. aureus* and prevent adherence. Mammalian skin and epithelia cells also secrete antimicrobial peptides such as defensins that can kill invading organisms (15). Once this primary defence is breached, bacterial components trigger activation of multiple components of the innate and adaptive immune response. Inflammation involves an orchestrated response from neutrophils, macrophages, endothelial cells, the complement system, the coagulation system, and the cytokine network. The neutrophil provides the cornerstone of a successful immune response against *S. aureus*. This is exemplified by the high incidence of *S. aureus* infections in patients with congenital neutrophil defects (142). Circulating neutrophils follow chemotactic signals to the site of *S. aureus* invasion. Chemotactic factors include components of the activated complement cascade (C5a), cytokines (Interleukin 1), or bacterial factors (formyl methionine peptides). Integrins, such as LFA-1 (lymphocyte function associated molecule), on the surface of neutrophils bind to selectins, such as ICAM-1 (intercellular adhesion molecule), on the endothelial cells and facilitate extravasation of neutrophils from the blood vessels into the tissue. The main function of activated neutrophils is phagocytosis. This is facilitated by opsonisation, whereby the inflammatory mediators label the bacterial surface for targeting by neutrophils and macrophages. The most important opsonins are C3b, generated during activation of the complement cascade and specific antibodies directed against targets on the surface of *S. aureus*. The opsonin on the bacterium is recognised by a specific receptor on the phagocyte and engulfed into a phagosome. The phagosome then merges with a lysosome, which is filled with powerful antibacterial chemicals and enzymes, and killing of the bacterium can occur.

It is well appreciated that *S. aureus* is adept at escaping from the host defences. The inflammatory response to bacterial infection is a complex interaction involving communication between various cells and plasma mediators. These interactions are coordinated by the cytokine network, in which there exists a delicate balance between pro- and anti-inflammatory effects. *S. aureus* produces various factors that disturb this balance, resulting in an uncontrolled activation of immune cells and mediators. The

best studied of these immunomodulators are the superantigens, notably enterotoxins A-J (114). The superantigens bind to the MHC Class II proteins on antigen presenting cells and cause massive uncontrolled cell proliferation and cytokine release. *S. aureus* also produces factors that specifically block chemotaxis (31) and neutrophil extravasation (21) (55). The majority of *S. aureus* strains produce a capsule under *in vivo* growth conditions and encapsulated strains are particularly difficult to opsonise. Protein A on the surface of *S. aureus* prevents opsonisation by binding antibodies in the incorrect conformation, such that an interaction between antibody and its receptor on the phagocyte cannot occur (142). Complement activation and various complement-mediated processes can also be blocked by *S. aureus* (83). If the bacterium is successfully phagocytosed it may still resist intracellular killing by producing sufficient levels of catalase and various peroxidases (114).

S. aureus pathogenesis

S. aureus is a well-armed pathogen, boasting a diverse array of virulence determinants. With the exception of the toxinoses (toxic shock syndrome, food poisoning, scalded skin syndrome), it is clear that no single factor is responsible for a particular staphylococcal infection and that development of infection is multifactorial. The organism stringently regulates virulence factors that have overlapping roles and can act either in concert or alone. Global regulatory elements that coordinate the expression of various groups of staphylococcal genes have been identified, such as *agr* and *sar* (3). A plethora of factors are implicated in pathogenesis, including factors involved in attachment, invasion, tissue penetration, and evasion of the host immune response. Attachment is mainly mediated by the extracellular matrix binding proteins (ECMBPs), discussed in the next section. *S. aureus* also produces a multitude of toxins. The cytolytic α-toxin has been shown to have a role in the development of certain infections (8). Enterotoxins A- J and Exfoliative toxins A and B have superantigen activity. Panton-Valentine leukocidin targets leucocytes and is associated with cutaneous infection (29). *S. aureus* also produces a variety of enzymes that destroy tissue, such as hyaluronidase, protease and lipase, and thereby facilitate spread to adjoining tissues. Another factor that may facilitate bacterial

spread is the production of staphylokinase, which forms a complex with human plasmin and activates the plasma fibrinolytic system (26).

A wealth of potential virulence factors are produced by *S. aureus.* It is clear that *S. aureus* must adjust to a variety of different environments in order to persist within the host and cause disease in diverse host tissues. The bacterial factors beneficial in causing infection in a superficial skin abrasion are, no doubt, very different to those required to survive within the bloodstream or establish infection in the lungs. A clear understanding of which factors or combinations of factors are necessary to cause a given infection remains elusive. Peacock et al. (112) have identified seven virulence determinants that are significantly more associated with *S. aureus* isolates from invasive disease than isolates from carriage. Whether these findings can be applied to diverse *S. aureus* populations remains to be determined. The general consensus is that any *S. aureus* strain can cause infection. Early reports that hypervirulent clones of *S. aureus* exist were later retracted (30) and a subsequent study from the same group concluded that there was no evidence for hypervirulent clones (46). It has, however, recently been reported that, while essentially any *S. aureus* genotype can cause infection, certain clones are more virulent than others (95). Rapid developments within the field of genomic profiling may provide the key to understanding whether or not hypervirulent clones with specific combinations of virulence determinants, in fact, exist.

Extracellular matrix binding proteins (ECMBPs)

In order to colonise and persist in the host, bacteria must adhere to host cells or the extracellular matrix. *S. aureus* produces surface proteins with specific affinity for host extracellular matrix and plasma components. These proteins are referred to as ECMBPs (Extracellular matrix binding proteins), MSCRAMMs (Microbial surface components recognising adhesive matrix molecules) (50), or Receptins (77). *S. aureus* produces one or more proteins that bind to human fibrinogen (Fg) (Table 1.2), fibronectin (Fn) (48) (69), collagen (Cn) (135), immunoglobulin (138) (162), keratin (105), prothrombin (Pt) (113), and von Willebrand factor (63), to name but a few. The interaction between *S. aureus* and these host proteins, may not only facilitate

colonisation of the host, but is also likely to disturb important cellular processes in which these ligands play a crucial role. This may be particularly beneficial for the bacterium and extremely detrimental for the host.

Fibrinogen binding proteins

The fact that *S. aureus* produces more than one fibrinogen-binding protein (FgBP) and that all strains have the ability to bind Fg, implies an interaction that is important for the bacteria. Since it is the most abundant host protein at the site of lesions, the interaction between *S. aureus* and Fg is considered to play a central role in the development of infections. Fg has been shown to be the most important adhesion promoting protein in *S. aureus* adhesion to biomaterials (141). Fg binding could also facilitate bacterial colonisation of damaged tissue and blood clots. Furthermore, coating the bacterial surface with host proteins, such as Fg, could facilitate evasion of the host immune response.

Table 1.2. Fibrinogen binding proteins from *S. aureus*

Protein	Location	Reference
ClfA	Cell surface	(92)
ClfB	Cell surface	(100)
Coagulase	Extracellular	(113)
FbpA	Cell surface associated	(22)
Efb (Fib)	Extracellular	(13)
FnBPA	Cell surface	(149)
Eap (Map)	Extracellular	(107) (94)
Emp	Cell surface	(66)
IsdA (Seg7, FrpA, StbA)	Cell surface	(23)

Clumping factor

Clumping factor A (ClfA) is the archetype member of a family of Gram-positive cell wall associated proteins (50). These proteins contain an LPXTG motif in the sorting signal which targets polypeptides for sortase A mediated covalent linkage to the cell wall. ClfA is the dominant protein responsible for the interaction between Fg and *S. aureus*. When ClfA binds to plasma Fg a complex is formed, which results in the "clumping" phenomenon that has been associated with pathogenic *S. aureus* since the turn of the last century (93). Fg is a dimeric protein that is converted by thrombin to form fibrin, the essential component of blood clots. Each Fg monomer consists of an Aα, Bβ and γ-chain (52). ClfA binds to the C-terminus of the γ-chain of fibrinogen (92). A second clumping factor, ClfB, has also been identified (100). ClfB and ClfA have the same overall organization and a great deal of sequence homology. In contrast to ClfA, ClfB interacts with both the α- and β- chains of Fg. Furthermore, *clfA* is expressed *in vitro* at all stages of bacterial growth (92), while *clfB* is only expressed during the early logarithmic stage (100). Comparison of ClfA producing strains to isogenic mutants has established an essential role for ClfA in the development of experimental endocarditis (98). This was confirmed when the *clfA* gene was inserted into the relatively non-pathogenic species *Lactococcus lactis*, resulting in enhanced pathogenesis of *L. lactis* in an experimental endocarditis model (115). An essential role for ClfA in septic arthritis has also been described (70). ClfA may also be the target for a protective immune response in *S. aureus* induced mastitis (125). The importance of ClfA in *S. aureus* infection is further underscored by the fact that passive immunization with anti-ClfA immunoglobulin provided some protection in a mouse sepsis model and a rabbit infective endocarditis model (58) (143).

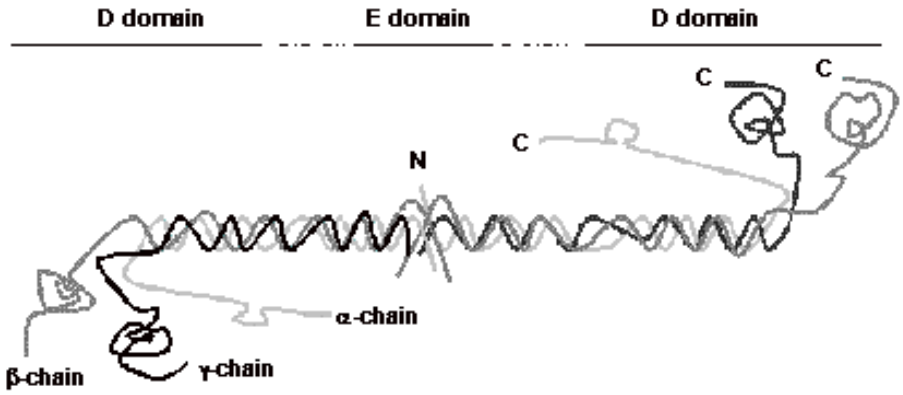

Figure 1.1. Schematic representation of Fg structure. The dimeric molecule is composed of three polypeptide chains, arranged such that all six amino termini are located in the central part of the molecule (E domain).

Fibronectin binding proteins

S. aureus expresses either one or two Fibronectin binding proteins (FnBPs), FnBPA or FnBPB, each containing the LPXTG motif. Fibronectin is found in a soluble form in plasma and as an insoluble component of the extracellular matrix, and is therefore abundant at sites of *S. aureus* colonization. While *S. aureus* has long been considered to be an extracellular organism it is now appreciated that it possesses the ability to invade eukaryotic cells (106) (1) (153) (18). The FnBPs significantly contribute to *S. aureus* internalisation *in vitro* (127) (38) (51). The interaction between the FnBPs and *S. aureus* is important in the pathogenesis of infection (96), particularly osteomyelitis (68). FnBPs may play an important role in the development of endocarditis, however, conflicting findings as to their relative importance have been reported (81) (47) (115). This likely reflects the multifactorial nature of pathogenesis, where various complementary binding functions play a role. FnBPA has also been shown to contain a distinct fibrinogen-binding domain in the N-terminal region (149). The importance of this interaction in a *clfA, clfB* positive background remains to be established.

Coagulase

Production of coagulase is traditionally used to distinguish pathogenic staphylococci, epitomised by *S. aureus*, from its less pathogenic relatives, epitomised by *S. epidermidis*. While the majority of *S. aureus* strains produce coagulase, a clear role for the protein in the pathogenesis of infection has yet to be established. It is produced *in vitro* in the exponential growth phase and the majority of the protein is extracellular. Coagulase contains a distinct Fg-binding (C-terminus) (12) and prothrombin-binding domain (N-terminus) (73). Coagulase binds to Pt and forms a complex at a 1:1 molar ratio, resulting in non-enzymatic conversion of plasma Fg to fibrin i.e. plasma clotting (62). The advantage to the bacteria of stimulating plasma clotting remains to be elucidated. It has been speculated that the generation of fibrin would enclose the bacteria in a fibrin capsule, thereby protecting them from the host's immune system. Virulence studies using isogenic mutants in animal models have shown no role for coagulase in subcutaneous and intramammary infection (113), but a potential role in the development of blood borne staphylococcal pneumonia has been proposed (121).

Broad spectrum binding proteins

Extracellular adherence protein (Eap), Extracellular matrix protein binding protein (Emp) and Iron responsive surface determinant A (IsdA) are broad spectrum binding proteins that include Fg in their binding repertoire. Emp is closely associated with the cell wall and binds to Fn, Fg, Cn, and Vitronectin (Vn), but a specific contribution to virulence has not been defined (66). IsdA is expressed on the cell surface under iron limited conditions and binds iron heme, transferrin, Fn and Fg (23).

Eap has been reported to bind to at least seven plasma proteins, including Fg, Fn, Pt, and Vn (94) (107). Maximal production occurs during the late exponential phase of growth *in vitro*, with 70% of Eap secreted into the culture supernatant. Eap contributes to the internalisation of *S. aureus* into epithelial cells and fibroblasts (56). The intracellular niche may provide protection from the host immune response. An anti-inflammatory role for Eap has also been described. Eap binds to intercellular

adhesion molecule 1 (ICAM-1) on endothelial cells, thereby inhibiting leucocyte adhesion and extravasation (21). Eap has also been shown to interfere with T-cell responses, disrupting the balance between the antibody (Th2) and cell-mediated (Th1) responses (84). Recently, Eap has been shown to have an intriguing dual effect on T- and B-cells. Low concentrations of Eap stimulated T-cell proliferation, while high concentrations inhibited proliferation by inducing apoptosis in T- and B-cells (57). The crystal structure of Eap has been solved and reveals considerable homology to the structure of superantigens from *S. aureus* and *S. pyogenes,* suggesting that Eap belongs structurally to a family of bacterial immunomodulators (54). Based on these *in vitro* findings, it seems that Eap represents an important immunomodulatory factor for *S. aureus*

Extracellular fibrinogen binding protein, Efb

Efb was first identified as one of three extracellular proteins purified from the supernatant of *S. aureus* Newman (11) (12). The authors reported a 19 kDa fibrinogen binding protein that was constitutively produced. While the majority of the protein was extracellular, a proportion remained associated with the cell surface. It was initially named Fib (13) but has since been renamed Efb and the molecular weight determined to be 15.6 kDa. Sequence analysis revealed that the N-terminus of Efb contains two nearly identical repeat regions, which have partial homology with the five to seven repeats at the C-terminus of coagulase (13). Efb was characterised at the DNA, RNA and protein level in *S. aureus* strains isolated from both carriage and infection (14). Efb was associated with almost 100% of *S. aureus* isolates, but was not found in coagulase negative species. Peacock et al have since reported an incidence of 60% for Efb in carriage isolates and 68% in isolates from invasive disease (112). Other studies have reported an incidence of 80% in human nasal and infection isolates (136) or 100% in rabbit infection isolates (140).

The predicted structure of Efb shows that the C-terminus contains substantial α helix while the N-terminus has a non-ordered structure (146) (85). The interaction between Efb and Fg has been well studied. Early work showed that Efb binding to Fg was

divalent, with one binding site in the N-terminal repeats and one in the C-terminal domain (109). Recently, Lee et al have shown that only the N-terminal region binds to Fg and no binding domain exists in the C-terminus (85). Both of the N-terminal repeats can bind to Fg with the second repeat showing slightly higher affinity (147). The combination of Efb and Fg in equimolar amounts leads to the formation of an Efb-Fg precipitation complex *in vitro* (109). This precipitation is clearly distinguishable from fibrin polymerisation mediated by coagulase. Efb has been reported to spontaneously form dimers (12) and this dimerisation may contribute to the formation of the precipitation complex with Fg.

In order to address the role of Efb in *S. aureus* infection, an Efb negative strain was generated using allele replacement (108). Lack of Efb did not affect the ability of the whole bacteria to bind to immobilised Fg. This is to be expected since Efb is an extracellular protein and the dominant surface bound FgBP has been clearly shown to be ClfA. Lack of Efb did not affect the pathogenesis of *S. aureu*s in a rat endocarditis model. An important role for Efb in *S. aureus* wound infection was determined when the Efb negative strain was compared with the isogenic parent strain in a rat model. Infection with the Efb producing strain was associated with significantly more severe wound infections, which healed more slowly.

Efb is produced *in vivo* during *S. aureus* infection, exposed to the host immune system and antibodies are produced. The antibody response to Efb in sera was compared between healthy individuals and those in the acute and convalescent phase of *S. aureus* septicaemia (28). Patients with acute *S. aureus* septicaemia had less anti Efb than healthy individuals but the level of anti Efb increased in convalescent sera. This suggests a potential protective role for antibodies against Efb. It could be postulated that it is the low Efb antibody levels that resulted in illness and the ability to produce these antibodies that leads to recovery. A potential protective role for antibodies against Efb has been shown in a mouse mastitis model (89). The animals immunised with a combination of coagulase and Efb prior to challenge were protected from the development of severe mastitis. The immunised animals developed only

mild infection and bacterial recovery was significantly lower from the site of infection in this group as compared with control animals.

Recently, a novel anti-inflammatory role has been ascribed to Efb. Efb has been shown to bind to C3b of the complement pathway and inhibit the classical and alternative pathway of complement activation (83). The region on Efb responsible for complement binding is in the C-terminus of the protein (85). Since Efb blocks a crucial central mediator of the complement system the entire cascade will be affected. This includes the generation of opsonins, chemotactic factors, and the bactericidal membrane attack complex. This establishes Efb as a potent inhibitor of the innate immune response.

S. aureus vaccine development

The search for a vaccine against *S. aureus* infection has been on going since the early 1900s. There has been little success achieved to date with such classical strategies as killed whole bacteria and various toxoid preparations. It is well appreciated that antibodies against *S. aureus* are prevalent in human sera. These may occur as a result of natural exposure to *S. aureus* in the normal flora or as a result of previous infection. It is clear that these antibodies are not protective since re-infection with *S. aureus* can occur throughout the individual's lifetime. In order to establish worthy targets for vaccine development it is imperative to identify antigens that generate a protective antibody response. In various animal models a protective antibody response has been characterised against ClfA (70) (143) (58), Collagen binding protein (102), the FnBPs (90) (17) (116), and Efb/coagulase (89). A protective antibody response against the capsular polysaccharides of *S. aureus* has also been described (44) (82). A polysaccharide conjugate vaccine has shown some success in clinical trials in haemodialysis patients (45). ClfA is the target for a humanized monoclonal antibody that provided protection in a mouse sepsis model and a rabbit endocarditis model (110). It is currently involved in a clinical trial for the treatment of *S. aureus* bacteraemia in conjunction with antibiotics.

S. aureus infection involves multiple virulence determinants and it is therefore unlikely that antibodies to a single component will provide adequate protection in a susceptible individual. The ideal vaccine would target several factors important at various stages of infection(s) and elicit antibodies that prevent adherence, promote phagocytosis and neutralise toxic components. Once the individual components of a multi component vaccine have been identified the next challenge to vaccine development rests in the identification of suitable target groups for either passive immunization or active immunization. The promiscuous nature of *S. aureus* as a pathogen complicates the identification of the patient groups that may benefit. Powerful proteomic techniques have been applied to identifying potential targets for protective antibodies (41) (145) (150). In this case it becomes extremely important to define what exactly constitutes a protective antibody. All three cited papers have used sera from infected patients to identify *in vivo* expressed antigens, which have triggered an antibody response. The difficulty with this type of screening rests in the assumption that these antibodies are protective. It is well appreciated that antibodies to *S. aureus* antigens in sera may not provide protection, since individuals become re-infected despite their presence. The question remains as to whether serology can be used to successfully identify vaccine candidates for a component of the normal flora, which is constantly exposed to the immune system. The possibility also exists that these antibodies could provide protection if sufficient levels are produced. In the case of *S. aureus* there remains an open question as to whether the lack of a protective immune response is due to the quantity or the quality of antibodies produced.

Platelets

In the course of infection *S. aureus* interacts with a variety of host plasma factors and cells, including platelets. Platelets, anucleated cells, patrol the bloodstream and inspect the integrity of the blood vessels. Platelets are central to the maintenance of vascular integrity and the prevention of bleeding from wounds. A simplified schematic overview of the role of platelets in haemostasis is shown in Figure 1.2.

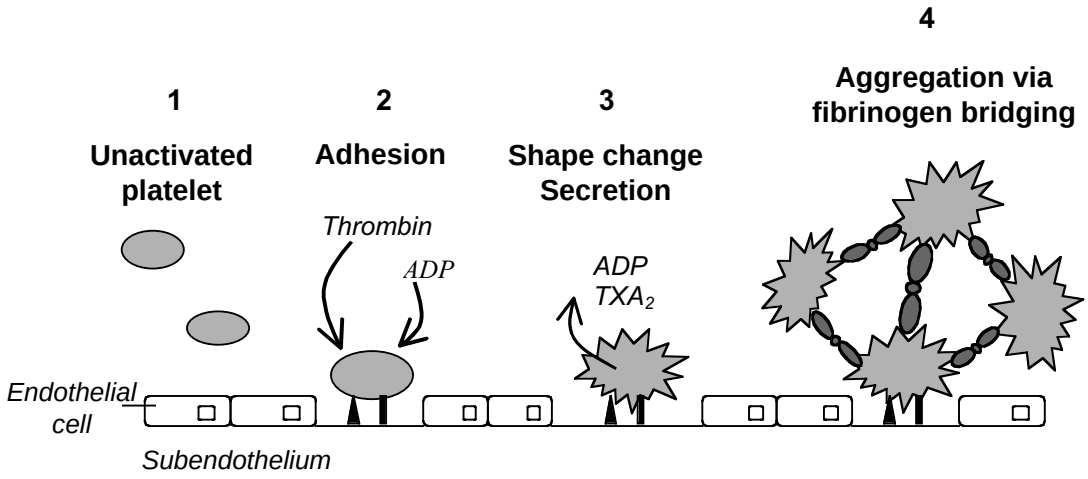

Figure 1.2. Platelets in haemostasis: Activation, adhesion and aggregation. 1. Unactivated platelets circulate in the bloodstream. **2**. When they recognise a site of tissue injury they adhere to exposed components of the subendothelial matrix; collagen (black triangle), vonWillebrand factor (black rectangle). Platelets can also be activated by thrombin, generated by the plasma coagulation cascade, and Adenosine diPhosphate (ADP), released from damaged endothelial cells. **3**. Activated platelets undergo shape change and release their granules, including the platelet activators ADP and Thromboxane A_2 (TXA_2) **4**. Various integrin receptors on the surface of platelets obtain an active conformation and can now bind their plasma ligands. Plasma fibrinogen binds to GPIIb/IIIa on adjacent platelets and forms a bridge between them. In this way platelet aggregation is mediated. The growing thrombus will then protect the integrity of the endothelium and prevent blood loss from the site of damage.

Platelets in circulation are normally maintained in an inactive state by prostacyclin and nitric oxide, released by endothelial cells (25). At sites of vascular damage they become exposed to components of the sub-endothelial matrix. The main matrix components involved in activation and adherence of platelets are various types of collagen and von Willebrand factor (vWF) (119). The initial tethering of the platelet

is mediated by specific platelet receptors interacting with vWF or Cn. Platelets are also activated by secreted mediators, which are abundant at the site of tissue injury. Potent platelet activators include thrombin, generated during activation of the coagulation cascade, and Adenosine diPhosphate (ADP), released from damaged endothelial cells and activated platelets (120). The various platelet agonists bind to distinct platelet membrane receptors, ultimately triggering intracellular signalling pathways. The activated platelet undergoes a shape change and granule release occurs. Platelets contain alpha and dense granules, which are an important source of adhesion molecules, growth factors, coagulation factors, and cell activating agents. Activated platelets release ADP from the dense granules and Thromboxane A2 (TXA_2) from immediate biosynthesis. In this way the platelet itself can activate other platelets in the area and the cycle becomes self-perpetuating.

Central to platelet activation is the modulation of the integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) into a high affinity binding site for Fg (123). Each Fg monomer has three potential binding sites for GPIIb/IIIa, two RGD containing motifs on the α chain and one binding site on the carboxyl terminus of the γ chain (7). There is a wealth of conflicting data with regard to the exact contribution of each region to Fg binding to GPIIb/IIIa (59) (74) (53) (75). The overall importance of the γ chain is illustrated in the study of Farrell et al using recombinant Fg in which the RGD and relevant γ chain motifs were mutated (43) (42). The current belief is that the γ chain region is the most important in platelet binding and aggregation and that the α chain is neither necessary nor sufficient. Since Fg is a dimer (Figure 1.1) it can essentially act as a bridge linking platelets together via binding of one Fg to the active conformation of GPIIb/IIIa on the membrane of neighbouring platelets. In this way the platelets become adhesive for one another and a thrombus is built up.

Concomitant to platelet aggregation, the coagulation system is activated in plasma and the end result is the generation of thrombin, which converts Fg to fibrin. Fibrin threads enmesh platelets, plasma components and blood cells to form the stable clot. A subsequent process of platelet retraction then occurs and culminates in drawing the edges of the wound together, reducing bleeding and supporting the healing process. Activated platelets are a rich source of growth factors, such as PDGF (platelet derived

growth factor) or TGF-α and β (transforming growth factor-α and β) involved in wound healing (91) (40).

Platelets are also important inflammatory cells with a significant role in host defence. They are capable of binding and internalising microorganisms (160). Platelets also express various receptors associated with immune functions, particularly complement receptors (CR3) (157). They have been shown to be capable of responding to cytokines and can also secrete cytokines (148). Perhaps the clearest host defence role rests in the secretion of an array of microbicidal peptides (tPMPs) by stimulated platelets. PMPs have been shown to have potent microbicidal effects on various microorganisms *in vitro*, such as *S. aureus, Candida albicans, Escherichia coli, and Cryptococcus neoformans* (158). The importance of tPMPs in inflammation is implied in the findings that *in vitro* resistance of *S. aureus* strains to PMPs correlates with more extensive disease in experimental endocarditis (35) (34). Resistance of *S. aureus* strains to PMPs *in vitro* also correlates with the ability to cause endocarditis (156). The relationship between *S. aureus* and platelets is far more complex than simply an antibacterial cell versus an invader and will be discussed in more detail in the next section.

Antiplatelet therapy

Platelet activation is tightly regulated, however a platelet on patrol cannot distinguish between various types of tissue lesions. When it reacts to inappropriate stimuli, a protective response becomes transformed into a potential disease causing mechanism. Inappropriate activation of platelets is of central importance in the development of cardiovascular disease, including coronary or peripheral arterial disease and arteriosclerosis (67). As such, the platelet represents an ideal target for treatment strategies in cardiovascular disease. This field of therapy has been revolutionised by such antiplatelet agents as aspirin and clopidogrel. Both drugs essentially block platelet activation, aspirin targets thromboxane synthesis and clopidogrel is an ADP receptor antagonist (10). The complex process of platelet activation and aggregation offers a wealth of potential targets for antiplatelet therapy. Activation of the integrin GPIIb/IIIa is central to platelet aggregation and as such represents an excellent target

for antiplatelet agents. The humanised monoclonal antibody abciximab (ReoPro) (27) is the archetype of the class and has had considerable success as an intravenous agent in percutaneous coronary intervention (9). The search for novel antiplatelet agents continues, since those currently available have problems related to efficacy and safety.

S. aureus and Platelets

S. aureus interacts directly and indirectly with human platelets. The whole bacterium can bind platelets and stimulate platelet aggregation *in vitro*, in the absence of other agonists (24) (159). *S. aureus* produces a number of surface proteins that are capable of binding to extracellular proteins, such as Fg, fibronectin and thrombospondin. Platelets also bind these proteins, therefore they could act as bridging molecules linking platelets and bacteria together and stimulating non-physiological platelet aggregation. It has been clearly shown that Fg is essential for *S. aureus* binding to and aggregation of platelets *in vitro* (64) (6). To date, several *S. aureus* surface proteins involved in bridging this interaction have been identified; ClfA, Protein A, FnBPA and FnBPB (104) (128) (61). A direct interaction between *S. aureus* and platelets has also been reported. ClfA has been reported to bind directly to a novel 118-kDa platelet membrane protein (126). Protein A binds to gC1qR, a complement receptor expressed on the surface of activated platelets (99). *S. aureus* α-toxin has also been shown to target platelets, stimulating procoagulant activity and thereby promoting coagulation (2). Thus, *S. aureus* has multiple mechanisms for binding to platelets leading to co-aggregation with platelets. This functional redundancy suggests an interaction that is important for the bacteria. Indeed, various other pathogenic bacteria have also been shown to bind to and aggregate platelets, including *Streptococcus sanguis* (134) (133) (49), *Enterococcus faecium*, *Enterococcus faecalis* (139), and *Streptococcus pyogenes* (80).

In endocarditis the platelet binding phenotype of the *S. aureus* strain is predicted to be a crucial factor in the pathogenesis of infection (132). A *S. aureus* strain with a diminished platelet binding capacity was significantly less virulent in experimental infective endocarditis (I.E). Furthermore, in I.E. anti-platelet agents, such as

acetylsalicylic acid, can cause substantial reduction in vegetation weight (101). It is well established that acetylsalicylic acid (ASA) is an antiplatelet agent, however an antibacterial effect has also recently been described (79) (78). ASA may therefore have a dual effect as a candidate treatment for *S. aureus* endocarditis. The importance of the stimulation of platelet aggregation by bacteria remains to be directly established *in vivo*. It is clear that platelets have an intriguing dual function in *S. aureus* endocarditis: adhesion promotion and antibacterial defence, in the form of antimicrobial peptides. The pathogenesis and severity of I.E. may largely be determined by the interplay between these paradoxical roles of platelets.

A wealth of data has been generated on the ability of *S. aureus* to stimulate platelet aggregation, however some antiplatelet effects have also been described. Lipoteichoic acid is associated with the membrane of *S. aureus* (155). It is one of the main components implicated in activation of the massive inflammatory response associated with septic shock (32). Recently, Lipoteichoic acid has also been shown to be a potent inhibitor of platelet aggregation *in vitro* (124). This antiplatelet effect is postulated to contribute to the bleeding abnormalities associated with *S. aureus* septic shock.

2. AIMS OF THE PRESENT STUDY

The overall aim of this study was to elucidate the biological role of Efb secreted by *S. aureus*. We specifically set about to:

- Identify the binding region on Fg for Efb.
- Determine the functional consequences of the sequence homology between the repeat regions of Efb and coagulase.
- Determine the effect of Efb on platelet function *in vitro*.
- Confirm the antiplatelet effect of Efb *in vivo*.
- Purify and characterise antibodies against Efb and assess the potential of Efb as a vaccine candidate.

3. MATERIALS & METHODS

The materials and methods used in this work are described in detail in the original papers, Appendix I - IV. In this section I will discuss some of the methods that are central to the thesis.

Protein purification

Native Efb was purified from the supernatant of an overnight culture of *S. aureus* Newman. The FgBPs present in the supernatant were purified using affinity chromatography over a Fg-sepharose column. Bound proteins were eluted with 0.7% acetic acid and dialyzed against 40 mM phosphate buffer pH 6.5 (PBS). The FgBPs were further purified by Fast protein liquid chromatography (FPLC) on a Mono S column. Three peaks, corresponding to coagulase, Efb and Eap, were eluted using a gradient of 0 to 1 M NaCl. The eluted proteins were then dialyzed to PBS, prior to storage.

GSTRR is a recombinant protein comprising the two repeats (amino acids 8-69) from the N-terminal part of Efb fused with GST (109). The combination of equimolar amounts of Efb and Fg leads to the precipitation of the Efb-Fg complex, which may interfere with the interpretation of results (109). GSTRR was therefore chosen for experiments carried out in a Fg-rich, plasma environment, since it represents one of the binding sites on Efb for Fg and does not precipitate with Fg. GSTRR was affinity-purified from *E. coli* BL21 cells using the procedure recommended by the supplier of the GST system (Pharmacia).

Efb210 is a recombinant protein containing the C-terminal part of Efb (amino acids 70 - 136), fused with an affinity tag of 6 consecutive histidines (13). Efb210 was affinity-purified from *E. coli* M15 cells using the procedure recommended by the supplier (Qiagen).

GST is a recombinant protein that was chosen as the negative control for all experiments in which GSTRR was tested. It represents the ideal control since it is purified from the same host organism as GSTRR. GST was affinity-purified from *E. coli* BL21 cells using the procedure recommended by the supplier of the GST system (Pharmacia).

Platelet isolation

Platelets were isolated from blood samples by centrifugation. Blood donors were healthy members of staff who had not taken antiplatelet medication, particularly non-prescription drugs containing aspirin, in the previous ten days. Five ml of blood was collected into citrated vacuum tubes (9:1 v/v into 3.8% trisodium citrate). Centrifugation at 140g for 10 minutes produced an upper platelet rich plasma (PRP), which was removed. The remaining lower phase was further centrifuged at 2000g for 20 min to obtain platelet poor plasma (PPP). The platelets in PRP were used within 1 to 4 hours. Since platelets are easily activated gentle handling was essential at all times.

Flow Cytometry

Flow cytometry is based on differences in light scattering properties and on fluorescence signals of cells in flow in front of a laser beam. We have used flow cytometry in order to investigate the effects of Efb on the platelet phenotype. A wide variety of fluorochromes are available, including fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP), used in this study. The emission spectra is different for these three fluorochromes, therefore they can be analysed together in the same sample in various combinations. In our basic assay, platelets in PRP were resuspended in Hepes buffer either in the presence or absence of ADP, for activation. After incubation for five minutes at room temperature the cells were probed with the fluorescent-labelled antibody or Efb. The labelling reaction was terminated after 10 minutes on addition of 1% paraformaldehyde in ice cold PBS. The samples were analysed on a FACSCalibur flow cytometer (Becton Dickinson) with a live gate set on the platelet population. Data from 50,000 cells was analysed in the

logarithmic mode. The data generated from each sample was analysed using Cell Quest software (Becton Dickinson), using the isotype controls to define the negative proportion of the population.

The platelet population was identified based on its light scattering characteristics or binding of the platelet specific antibody, CD61 PerCP. The basic assay was used to study activation dependent binding of three different antibodies (anti Fg FITC, PAC-1 FITC, CD62 PE) to platelets in the presence of Efb or GSTRR. Flow cytometry was also used to investigate the binding of FITC labelled Efb or GSTRR to platelets. A basic flow chart of sample analysis is shown in Figure 3.1.

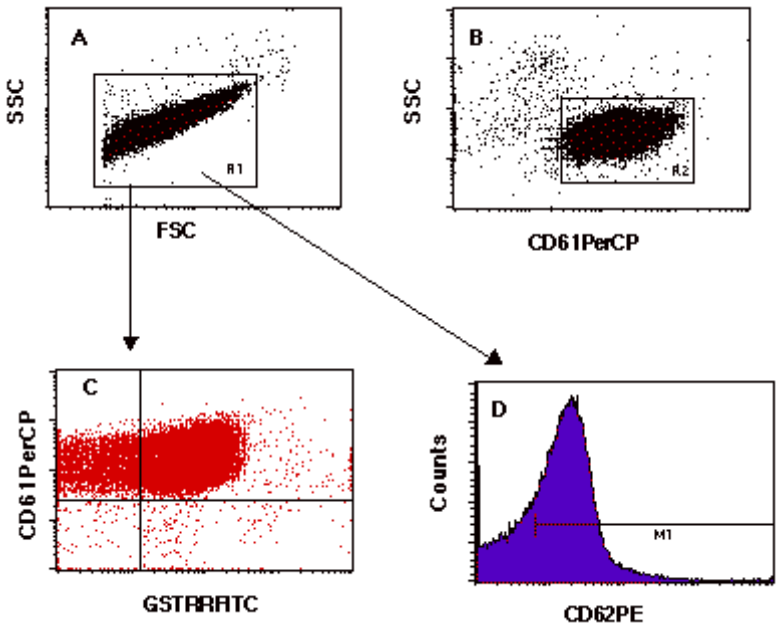

Figure 3.1.Analysis of platelets by flow cytometry. The platelet population is identified by its light scattering characteristics (A) or by binding of the platelet specific antibody CD61 (B). A region gate is drawn around the platelets, R1 or R2 in A and B, respectively. When double staining is carried out the cells from this region are analysed in dotplots (C) of fluorescent signal for channel 1 versus fluorescent signal for channel 2. When single staining is carried out the cells from the defined region are analysed in histograms (D) of fluorescent signal versus cell count.

4. RESULTS & DISCUSSION

Identification of the binding region on Fg for Efb

In order to determine the region of Fg to which Efb binds, we used Western affinity blotting and affinity chromatography on Efb-Sepharose with the purified chains of Fg. The individual chains of Fg (Aα, Bβ and γ) were separated from one another by preparative SDS-PAGE continuous elution electrophoresis. Equal amounts of each chain were combined with Efb-Sepharose and mainly the Aα chain was recovered under these conditions **(Paper I)**. This specific interaction was confirmed by Western blot, in which the three chains of Fg were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with Efb. Mainly the Aα chain was recognised by Efb **(Paper I)**.

Digestion of Fg with plasmin produces a set of fragments designated A-E. Fragments D and E are the largest fragments generated with masses of 50% and 15% of the Fg molecule, respectively. Fragment D corresponds mainly to the distal domain and E to the central domain. We have purified Fragment D and have shown in an ELISA that Efb and GSTRR bind equally well to both complete Fg and to fragment D **(Paper I)**. This implies that the Efb binding site is conserved when Fg is degraded to fragment D. The isolated D fragment contained two closely migrating bands of 45 kDa (corresponding to fragments of the Bβ- and γ-chains) and a band of 14 kDa (from Aα-chain). In a Western blot the Efb probe recognised the Aα-chain of the D fragment **(Paper I)**.

Fragment D includes residues 111-197 of the Aα-chain (129). The RGD sequence (residues 95-97), which is recognized by the platelet integrin GPIIb/IIIa and may contribute to platelet aggregation, is located close to this region of the Aα-chain (59). Interaction between thrombospondin and Fg, which may also be important in platelet aggregation, takes place at residues 113 - 126 of the Aα-chain (4). Binding of Efb at or near these sites could have an effect on platelet function or other important physiological processes where such sites are involved. It was for this reason that we investigated the interaction between Efb and platelets.

Functional consequences of the homology between Efb and coagulase

Since the N-terminal repeats of Efb show some homology to the five to seven repeats of coagulase we set about to investigate any possible competition between the two molecules. In a capture inhibition ELISA, binding of Fg to GSTRR was decreased in the presence of coagulase **(Paper I)**. Coagulase exerts its biological effects through the formation of a complex with prothrombin that stimulates the conversion of Fg to fibrin, leading to the formation of a clot. Efb inhibited the coagulase activity of coagulase in plasma **(Paper I)**. Together with the partial sequence homology between these repeats, it strongly implies identical recognition sites on Fg for Efb and coagulase. This is further underscored by the finding that hyperimmune IgG against Efb is also capable of recognising coagulase. As a result of this cross reactivity, antibodies against Efb also recognise coagulase and block coagulase function **(Paper IV)**.

The interaction between Efb and platelets *in vitro*

Efb is a FgBP and as such it may interfere with various host cell functions in which Fg plays a role. Fg is a dominant plasma protein and plays a central role in blood coagulation, both in terms of clot formation and mediating irreversible platelet aggregation. In this study, we have characterised the effect of Efb on these Fg mediated events. To this end we have developed a number of flow cytometry based assays to assess platelet function in the presence of Efb. The platelet agonist used in flow cytometry was ADP. ADP was used at a concentration of 1 μM, as this represents half the maximal concentration for activation. GST was used as the negative control and tested in parallel in all experiments performed.

In order to investigate the direct interaction between Efb and platelets, whole native Efb or recombinant GSTRR was FITC labelled and incubated with platelets in the presence of a specific platelet marker, CD61PerCP. GSTRRFITC and EfbFITC bind to platelets, only after ADP activation (Figure 4.1). The specificity of this interaction is proven by the fact that preincubation of platelets with unlabelled GSTRR dose

dependently blocks GSTRRFITC and EfbFITC binding to platelets **(Paper II)**. The fact that EfbFITC binding to platelets was abolished by unlabelled GSTRR implies that the binding site on Efb for platelets is located within the N-terminal domain of Efb. This region of Efb was previously shown to bind to platelets using a phage display library of *S. aureus* and Fg was speculated to be acting as a bridging molecule between Efb and platelets (60). Indeed, Fg has previously been shown to be an essential bridging molecule in some interactions between *S. aureus* and platelets (64) (6).

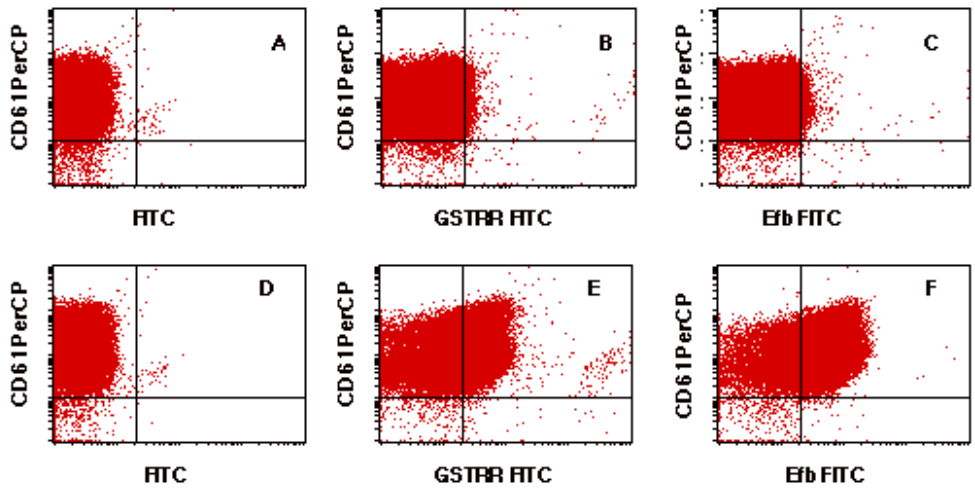

Figure 4.1. Efb binds to ADP activated platelets. A CD61 positive platelet population was gated and probed with various concentrations of FITC labelled Efb or GST-RR. Data is represented as dot-plots of FITC versus PerCP fluorescence intensity, generated by Quest software. Double positive cells appear in the upper right quadrant of the dot plot. These cells are positive for CD61PerCP, indicating that they are platelets, and FITC, indicating that they have bound Efb or GSTRR. EfbFITC (0.8 µM) and GSTRRFITC (1.2 µM) bind to platelets after ADP activation (E and F), while only background levels of EfbFITC and GSTRRFITC bind to unactivated platelets (B and C)).

In our study we have investigated the role of Fg in Efb binding to platelets and established that the predominant part of this binding occurs independently of Fg. When Fg depleted platelets were substituted for PRP, Efb and GSTRR could bind equally well to the platelet surface and reconstitution with biologically active exogenous Fg had no effect on the interaction **(Paper II)**. Fg depleted platelets do contain residual Fg, therefore we set about to confirm that GSTRR binding to platelets is independent of Fg in a system that is completely free of platelet bound Fg. Fg binds to the active conformation of the integrin receptor GPIIb/IIIa on platelets and this interaction can be blocked by the monoclonal antibody, ReoPro (27). In the presence of ReoPro, Fg binding to activated platelets was completely abolished. Despite the presence of ReoPro, GSTRRFITC still bound to platelets in a concentration dependent manner **(Paper II)**. However, there was an increase in the level of GSTRR binding when ReoPro was omitted from the system. This may reflect a fraction of GSTRR binding to platelets that is mediated by Fg and which can be blocked by ReoPro. It is clear that GSTRR can bind both directly to platelets and possibly to a lesser extent indirectly, mediated by Fg bridging. We have therefore demonstrated that Efb binds to activated platelets in a Fg independent manner to an as yet unidentified surface component.

In order to assess the consequence of Efb binding for the platelets, we took a panel of activation dependent platelet markers and used flow cytometry to determine their presentation in the presence of GSTRR. Fg binds to the integrin receptor GPIIb/IIIa on agonist-stimulated platelets and PAC-1 recognises the active conformation of this receptor (122). CD62 (P-Selectin) is released from the α-granules and presented on the surface of activated platelets (130). CD62 presentation by ADP activated platelets was significantly decreased in the presence of GSTRR. There was also a tendency toward inhibition of PAC-1 binding but this was not significant **(Paper II)**. This implies a general inhibition of platelet activation. In contrast, we found that Fg binding to platelets was stimulated in the presence of GSTRR **(Paper II)**. In order to elucidate this intriguing finding, we set about to characterise the nature of the Fg binding to platelets that occurs in the presence of GSTRR. Under normal circumstances Fg binds to the active conformation of the integrin receptor GPIIb/IIIa on platelets and this interaction can be blocked by the monoclonal antibody, ReoPro.

In the presence of ReoPro alone, Fg binding to activated platelets was completely abolished. However, when GSTRR or Efb was added to the system, Fg was once again capable of binding to platelets. ReoPro abolished the fraction of Fg binding that was mediated by the normal receptor, however ReoPro had no effect on the fraction that is mediated by Efb **(Paper II)**. This illustrates that Efb stimulates a novel type of Fg binding to platelets that does not involve the normal platelet receptor.

The results from the flow cytometry appear to be somewhat contradictory. On the one hand Efb stimulates Fg binding to platelets, while on the other hand the binding of activation dependent markers (CD62 and PAC-1) is decreased. In order to clarify the situation, we assessed the effect of Efb on platelet function in a more biological assay. Born aggregometry (16) was used to determine platelet aggregation *in vitro*. GSTRR and Efb were potent inhibitors of platelet aggregation, both in response to ADP and collagen mediated activation **(Paper I & II)**. These platelet agonists bind to different platelet membrane receptors and initiate various intracellular signalling events, fundamentally resulting in the modulation of the integrin GPIIb/IIIa into a high affinity/avidity binding site for Fg (123). The inhibitory effect of Efb on platelet aggregation is likely to involve a common step in both of these activation pathways. In response to activation, platelets undergo a variety of biochemical and morphological changes and the platelet shape change response is a crucial intermediary phase in platelet aggregation (118). Efb and GSTRR caused platelets to appear in flow cytometry dot plots as unactivated platelets, despite the presence of activators **(Paper II)**. The effect of Efb and GSTRR on platelet shape reflects the inability of platelets to aggregate in the presence of Efb or GSTRR. This establishes Efb as an inhibitor of platelet activation and aggregation.

Efb binds to an activated platelet and inhibits activation and aggregation. We propose that this inhibition is mediated by the novel Fg binding to the platelet surface that occurs via Efb bridging. A model summarising the various interactions between Efb and platelets described in this study is illustrated in Figure 4.2. Efb binds to an activated platelet, either directly to receptor x (Figure 4.2C) or to a lesser extent via Fg bridging (Figure 4.2B). Plasma Fg then binds to the Efb on the platelet surface

(Figure 4.2D). It is this Efb mediated binding that is responsible for the novel binding of Fg to platelets, which we observed in the presence of Efb. Fg bound via Efb is obviously held in a non-functional conformation since platelet aggregation is inhibited. Platelet aggregation is essentially dependent on Fg cross-linking platelets, since as a dimeric molecule, one Fg can bind to two platelet cells simultaneously and also partake in cross bridging interactions with various plasma components. It may be that Fg bound via Efb is sterically hindering or blocking these various cross bridging interactions. It is also possible that the intracellular signalling events orchestrated by the integrin GPIIb/IIIa, which are central to generating irreversible platelet aggregation (87), are negatively affected by Efb bound to the platelet surface.

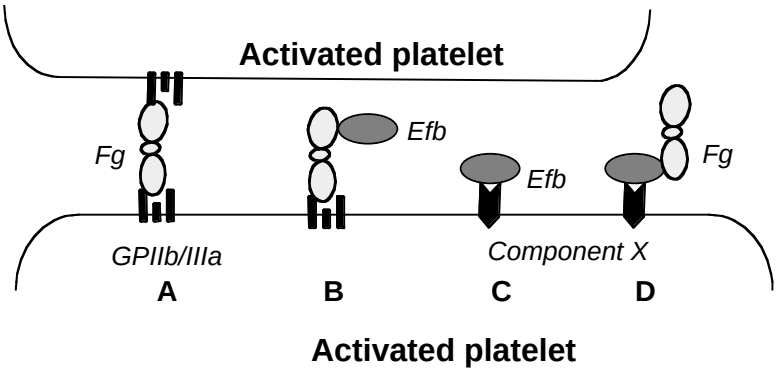

Figure 4.2. Schematic representation of the proposed interactions between Efb and platelets. Under normal circumstances Fg binds to the GPIIb/IIIa complex on activated platelets, forming a bridge with other platelets (A). We propose that Efb has at least two mechanisms of binding to platelets, either via Fg bound to the platelet surface (B) or directly to the platelet surface via an as yet unidentified receptor, designated X here (C). Fg may in turn be able to bind to the platelet bridged by Efb bound to Component X (D).

Efb as an antiplatelet agent *in vivo*

Having established that Efb is a potent antiplatelet agent *in vitro*, the next step was to evaluate if this is also the case in the complex environment within the host. Inbred female Balb/c mice were used for all experiments. An inbred strain was chosen in order to minimalise the inter-animal variability, likely associated with a more heterogeneous genetic background. Haemostasis in the mouse has been well studied and it is strikingly similar to the human system. Mice have higher platelet counts than humans and their platelets have a shorter life span. Fg and GPIIb/IIIa are required for

platelet aggregation in mice and aggregation in response to ADP and Cn is comparable to that for human platelets (137).

As discussed in the introduction, fully functioning platelets are required in order to prevent bleeding from a site of tissue damage. In order to assess platelet function *in vivo* in the presence of GSTRR, a bleeding time assay was developed. This is largely based on the work of Dejana et al in rats (33). GSTRR significantly prolonged the bleeding time and the effect was relatively short lived, returning to normal approximately three hours after administration of GSTRR **(Paper III)**. GST, as a negative control, had no effect on bleeding time. The time to cessation of bleeding is determined by the ability of platelets and plasma factors to form a stable plug at a site of tissue injury. It was therefore important to demonstrate that GSTRR had no effect on the coagulation cascade ex vivo **(Paper III)**. Bleeding time was prolonged, despite a normal coagulant status, therefore it is clear that Efb specifically inhibits platelet function. Efb has the same effect on platelets *in vivo* as it has on isolated cells *in vitro*, therefore Efb directly targets platelets and does not mediate an effect via other cells as mediators.

The ability to prevent blood loss from damaged tissue reflects the physiological function of platelets, however platelets also have a central role in pathological thrombosis. The effect of GSTRR in a mouse model of acute thrombosis was therefore evaluated. The mouse thrombosis model is adapted from DiMinnio et al (36). The model is of particular value for screening antithrombotic agents that work primarily by inhibiting platelet aggregation and agents that target the coagulation system are ineffective in this model. The cause of death is primarily due to occlusion of the microcirculation in the lungs by platelet thrombi. Thrombosis is induced by an intravenous injection of a combination of the powerful platelet activator Cn and the potent vasoconstrictor epinephrine. Within three to five minutes of Cn/epinephrine administration, all of the animals tested developed respiratory distress, hind limb paralysis and died. Survival times were compared between two groups of animals, pre-treated either with GSTRR or GST. GSTRR rescued all animals from death when administered immediately prior to thrombogenic stimulus and these animals survived

for two hours before being sacrificed **(Paper III)**. GST offered no protection. Remarkably, the inhibition of platelet function in the presence of GSTRR is so powerful that it can counteract massive thrombosis and resultant death.

The protective dose of GSTRR in the mouse model is equivalent to a dose of 20 mg/kg. This may appear to be a high dose but it is well within the range of required doses for other well-established antiplatelet agents tested in the same model (71) (161) (72) (97) (65). The model is based upon the exogenous administration of large doses of potent thrombogenic agents and as such does not closely mirror the normal events associated with a thrombotic episode in cardiovascular disease. The protective doses of test agents required in our mouse model are likely to represent overdosing in a more physiological system. It remains an excellent proof of principle test in which GSTRR, a powerful platelet inhibitor, is capable of protecting animals from death due to thrombosis. The model remains one of the most frequently used small animal models of thrombosis. In other small animal models endothelial cell damage is induced by various physical and chemical stimuli (37) and these models offer few advantages over our chosen model. Large animal models provide a system that more closely mimic the *in vivo* events during clinical thrombosis since isolated segments of vessels can be more easily manipulated and monitored. Evaluation of Efb in large animal models is the next step for assessment of its antiplatelet effects.

Inhibition of platelet function – implications for *S. aureus* pathogenesis.

We have clearly demonstrated that Efb inhibits platelet function, but what then are the implications for the infected host? As discussed in the introduction, platelets are essential for haemostasis, contribute to wound healing and are a source of inflammatory mediators. Efb, as a powerful antiplatelet agent, may affect any or all of these platelet functions. In this study we have elegantly shown that Efb impairs haemostasis and platelets can no longer efficiently prevent blood loss from a site of injury. This neither implies nor requires that excessive bleeding on a systemic level should be a symptom of *S. aureus* infection. The quantity of Efb produced during

infection can only be speculated but it would not be expected to reach systemic levels at which bleeding abnormalities would occur. During infection we must consider local effects in the microenvironment in which the bacteria have established themselves, typically an abscess. Locally, at the site of infection it is likely that Efb is produced in adequate amounts to inhibit platelet function.

S. aureus is a significant cause of wound infection and a hallmark of infected wounds is a compromised wound healing response (39). It has previously been shown that Efb contributes to *S. aureus* pathogenesis in a rat wound infection model (108). In this model, the virulence of an Efb producing strain was compared to an isogenic Efb mutant strain in wound infection. The animals infected with the Efb knockout mutant developed mild wound infection, while those infected with the Efb producing strain developed significantly more severe infections. The production of Efb in the wounds was associated with impaired wound healing. Furthermore, we have shown that antibodies against Efb protect against severe *S. aureus* wound infection in a mouse model **(Paper IV)**. Platelets are essential for normal wound healing and inflammation (40) (91), providing a scaffold for the infiltration of the various cells involved in the process and providing important cytokines and growth factors involved in the healing response. The retardation of healing associated with Efb is therefore explained by the inhibition of normal platelet function mediated by Efb.

As discussed in the introduction, an interaction between *S. aureus* and platelets has previously been established. The bacterium, via various ligands, can stimulate platelet aggregation. In this work we describe an inhibition of platelet function by a *S. aureus* secreted protein. It seems to be an intriguing contradiction for the bacterium to have such opposing effects on platelet function. It seems logical that both events do not occur simultaneously and that the bacterial ligands responsible are not expressed simultaneously. The expression of *S. aureus* virulence factors has been well studied in broth culture but this is a highly nutritious environment and may not reflect the situation during different stages of infection or different niches within the host. It is well appreciated that at different stages of infection, or in different types of infections, different strategies are employed by the bacterium and a stimulation of platelet

aggregation may be preferred over an inhibition of platelet aggregation or vice versa. For example, the stimulation of platelet aggregation is considered to be a central mechanism in the development of the infectious plaque in infective endocarditis (132). It has been demonstrated that Efb does not contribute to virulence in a rat endocarditis model (108). Conversely, the inhibition of platelet aggregation triggered by Efb contributes to the pathogenesis of *S. aureus* wound infections. This is not the only case of *S. aureus* producing factors with apparently opposing effects. Coagulase targets plasma prothrombin and stimulates fibrin polymerisation, while staphylokinase targets plasma plasmin and activates the fibrinolysis. Once again, from the bacterial point of view, it is highly implausible that both events occur at the same time.

Efb is the first bacterial protein to be identified that specifically inhibits platelet function. There are toxins that target platelets, such as alpha toxin from *S. aureus.* Paradoxically, this has been shown to stimulate procoagulant function and therefore coagulation (2). YopM from *Yersinia pestis* inhibits platelet aggregation (86). YopM binds and sequesters thrombin and prevents thrombin mediated platelet aggregation but aggregation in response to other activators has not been assessed. Efb represents a global inhibitor of platelet aggregation in response to diverse stimuli.

Efb in antiplatelet therapy

In pathological syndromes involving a failure of haemostasis the platelet represents a worthwhile target for the development of treatment strategies. The current state of the field of antiplatelet therapy was discussed in the introduction. Efb may represent a novel antiplatelet agent suitable for clinical evaluation. Indeed, bacterial factors have successfully been developed in the related field of antithrombotic therapy. Streptokinase from streptococcal species and staphylokinase from *S. aureus* have both been successfully used as thrombolytic agents for the treatment of acute myocardial infarction (5) (26). A major limitation associated with both agents is the development of neutralising antibodies in treated patients and efforts are being made to generate less immunogenic mutants. In a fascinating attempt to combine the fields of thrombolytic and antiplatelet therapy, a staphylokinase mutant has recently been

generated that both stimulates fibrinolysis and inhibits platelet aggregation *in vitro* (131). In order to develop Efb as an antiplatelet agent for clinical use it may be important to identify a less immunogenic short peptide, which retains antiplatelet function. We have carried out peptide mapping based on the N-terminal region of Efb (amino acids 1 – 69). Three peptides have been identified that bind Fg and mimic the effects of whole Efb on Fg binding to platelets, however we have to date not identified a peptide that can block platelet aggregation and prolong bleeding in mice (unpublished observations). It may be that short peptide fragments cannot inhibit platelet function because the structural integrity essential for this interaction is not maintained in such fragments.

Antibody responses against Efb

We have purified both hyperimmune antibodies against Efb and naturally occurring antibodies against Efb and tested their ability to neutralise selected biological effects of Efb. Immunization with purified Efb and Freunds adjuvant generated high titre specific antibodies, which we characterised *in vitro* and *in vivo*. We have shown that hyperimmune IgG against Efb neutralises the biological effects of Efb. In the presence of IgG against Efb, the binding of Efb to Fg is blocked, inhibition of platelet aggregation by Efb is counteracted and the retardation of wound healing associated with Efb is abolished **(Paper IV)**. The IgG generated in response to vaccination with Efb also cross reacts with coagulase and blocks coagulase function in plasma. Since the role of coagulase in *S. aureus* infection has yet to be clarified, we cannot rule out the possibility that a blockade of coagulase function contributes to the protection offered by hyperimmune IgG against Efb.

In the animal model, female NMRI mice were immunised with Efb in combination with Freunds adjuvant and subjected to a foreign body associated wound infection model (Figure 4.3). The results of this model give a clear indication of the role of Efb in *S. aureus* wound infection. A relatively low quantity of bacteria is used to generate infection and the animals are not severely compromised, as demonstrated by the lack of significant weight loss in both vaccinated and unvaccinated animals. The bacterial load recovered is the same from each group, therefore Efb does not contribute to the

initial colonisation of and persistence in the infected site. The difference between the two groups is related to the severity of infection and this difference is significant. The vaccinated animals tended to have only mild wound infections, which had healed or were healing well at the end of the experimental period **(Paper IV)**. The unvaccinated animals had far more severe infections that had failed to heal by the end of the experimental period. Once again, we see a retardation of wound healing in the presence of Efb. The survival advantage that this strategy offers to the organism is speculative but it is plausible that the bacteria are freer to spread from this niche to other sites in the body.

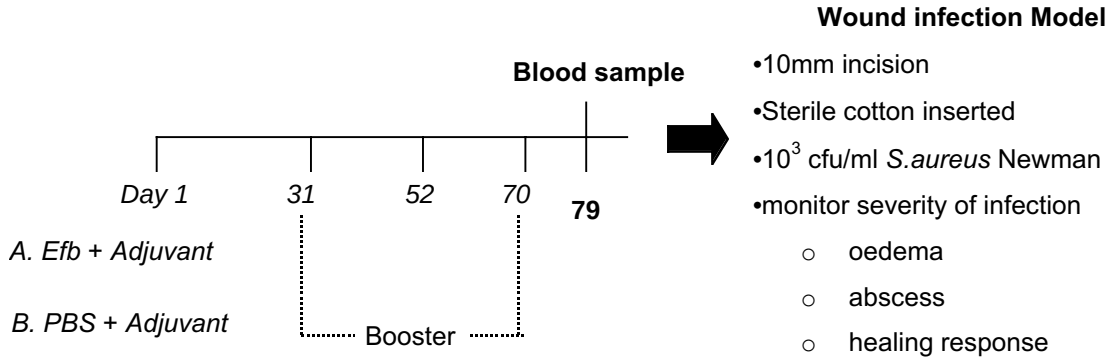

Figure 4.3. Immunization and foreign body associated wound infection model. Female NMRI mice were divided into two groups, A and B. Group A were immunised with Efb in complete Freunds adjuvant and Group B were immunised with PBS in complete Freunds adjuvant (control group). Both groups received a booster prepared in incomplete Freunds adjuvant, on day 31, 52 and 70. Blood samples were taken on Day 79 and tested for the presence of antibodies against Efb in ELISA. The animals in both groups were subjected to a foreign body associated wound infection model.

We have also found that specific IgG recognising Efb is present in commercial IgG pools from healthy people. This may be as a result of previous exposure to and recovery from *S. aureus* infection or simply due to carriage of *S. aureus*. Monospecific human IgG recognising Efb, specifically the N-terminal repeat region from human IgG pools, were purified. This naturally occurring human IgG failed to neutralise Efb and block its biological effects. The monospecific human antibodies failed to block the interaction between GSTRR and Fg, even at concentrations equivalent to the protective dose of polyspecific sheep antibodies **(Paper IV)**. In the case of the polyspecific IgG we can only estimate the fraction that recognises

GSTRR, while the monospecific human IgG represents the purified specific fraction of IgG recognising GSTRR. As such, the quantity of human IgG is unlikely to explain the inability of the human IgG to neutralise Efb. It is clear that there is a significant qualitative difference in the type of immune response generated by immunization with Efb, as compared to that generated by natural exposure to Efb. In this study we found that immunization with purified Efb and adjuvant is essential to generate protective antibodies against Efb.

We have established that Efb is a worthy target for the development of a protective antibody response in target patients. This could include patients with various open wounds, burn injuries, diabetic patients or those undergoing elective surgery. Efb is produced by the majority of *S. aureus* strains tested, with isolates showing little sequence variation (14) (136) (140). This makes it a good target as a vaccine candidate, since it is unlikely to cause serotype specific responses. It is well appreciated that the development of *S. aureus* infection is multifactorial. It was clearly shown in our wound infection model that Efb did not play a role in the initial stages of colonisation and establishment of infection but played a significant role in the late stage of infection, particularly persistence in the area. Therefore, it is not the only vaccine candidate and would alone be unlikely to offer protection against establishment of infection but could be used as part of a multi-component vaccine. The ideal vaccine would target several virulence factors important at various stages of infection(s) and elicit antibodies that prevent adherence, promote phagocytosis and neutralise toxic components. Based on the current knowledge, FnBPA or ClfA represent worthy targets for adherence blocking and opsonic antibodies and Efb is a target for neutralising antibodies. In the case of Efb, opsonic antibodies are unlikely to be generated since very small quantities of Efb remain associated with the cell surface. A toxin candidate for this multi component vaccine has not been identified and may not even be required. The fact remains that Efb represents a worthwhile candidate for active or passive immunization in certain *S. aureus* infections.

5. CONCLUSIONS

In this thesis we have characterised the role of the secreted *S. aureus* protein, Efb, in *S. aureus* infection. We have shown that Efb binds to activated platelets and mediates a novel type of fibrinogen binding to the platelet, which does not involve the normal integrin receptor for fibrinogen on platelets. As a result of this binding platelet activation is diminished. In the presence of Efb, the platelet shape change response and the presentation of activation dependent markers (CD62 & PAC-1) is decreased. Efb inhibits platelet aggregation in response to various platelet activators. Efb is therefore a powerful inhibitor of platelet function. We have also demonstrated that Efb inhibits platelet function *in vivo*. The tail bleeding time is significantly prolonged in the presence of Efb. Furthermore, in an animal model, intravenous Efb rescued 100% of animals from death due to acute thrombosis. This reflects the inability of platelets to function normally and aggregate in the presence of Efb.

An essential role for Efb in *S. aureus* wound infection has previously been described (108). The production of Efb in the wounds was associated with impaired wound healing. Platelets are essential for wound healing (40) and the retardation of wound healing associated with Efb in wound infection likely reflects the inhibition of platelet function characterised in this thesis. The importance of Efb in wound infection is underscored by the finding that immunization with Efb protected animals from the development of severe *S. aureus* wound infections. Antibodies generated in response to immunization with Efb were capable of neutralising Efb *in vitro* and blocking the biological effects of Efb. These antibodies blocked Efb binding to fibrinogen, neutralised Efb and returned platelet aggregation in the presence of Efb to normal. Efb represents a target for a protective antibody response and is therefore a worthwhile vaccine candidate for certain *S. aureus* infections.

In conclusion Efb is a powerful antiplatelet agent, which impairs haemostasis and wound healing in *S. aureus* infection. Efb also represents a worthwhile vaccine candidate for certain *S. aureus* infections.

6. POPULAR SCIENCE SUMMARY

Staphylococcus aureus are bacteria that are commonly carried on the skin or in the nose of healthy people. In many cases this is an innocuous event but these bacteria may also cause infection. *S. au*reus is a leading cause of infection in both the community and the hospital environment. Infection may manifest as a minor, self-limiting event or a serious infection requiring medical intervention. Treatment of these infections is becoming more complicated because the bacteria have developed resistance to diverse antibiotics. In order to combat these infections and develop new treatment strategies, it is important to understand what bacterial factors contribute to infection. In our study we have been working with a protein, Efb, secreted by *S. aureus*.

Efb was initially discovered on the basis of its ability to bind to human fibrinogen. Fibrinogen is an abundant protein in blood. In response to tissue injury, such as a cut, fibrinogen and small blood cells, platelets, act in concert to prevent blood loss from the area and heal the wound. In the course of our study we located the region on fibrinogen to which Efb binds, and found that this region is important for the interaction between fibrinogen and platelets. We therefore investigated the effect of Efb on platelet function. We have clearly shown that platelets are unable to function normally in the presence of Efb. We have demonstrated that after platelets have become activated, in response to tissue injury, Efb binds to the platelets. Efb diminishes normal platelet activation and prevents them from adhering to one another, so called aggregation. In the absence of platelet aggregation blood clot formation is inhibited and bleeding from the site of injury is prolonged. Furthermore, we found that the ability of wounds to heal properly is impaired in the presence of Efb. This is not surprising since functional platelets are required for wound healing.

The ability to prevent blood loss is a reflection of the normal function of platelets, however these cells also contribute to the inappropriate blood clotting (thrombosis) seen in some diseases. These diseases include myocardial infarction, stroke and arteriosclerosis. In our study we found that the inhibition of platelet function by Efb is

so powerful that Efb can prevent thrombosis. In an animal model Efb could rescue 100% of animals from death caused by thrombosis. This suggests a potential application of Efb outside the field of *S. aureus* infection. It may be possible to develop Efb into a new drug to treat diseases where inappropriate blood clotting occurs.

Having established the role of Efb as a platelet inhibitor in *S. aureus* infection, the next challenge was to determine if we could counteract these effects. We immunised animals with Efb in order to prime the immune system. They developed a good antibody response against the protein and these antibodies were able to neutralise Efb and protect the animals from a subsequent *S. aureus* wound infection. This means that a vaccine based on Efb may protect against the development of certain S. *aureus* infections in humans.

Overall in this thesis we have characterised the role of a secreted protein, Efb, in *S. aureus* infection and identified it as a potential target for the development of novel treatment strategies for these infections.

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Happiness is neither virtue nor pleasure nor this thing nor that, but simply growth, we are happy when we are growing.

-William Butler Yeats

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