LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

The Molecular Characterization of the Clumping Factor A (ClfA) from *Staphylococcus aureus* using Monoclonal Antibodies

A thesis submitted for the degree of Doctor of Philosophy

by

Gillian Mc Namara

Department of Microbiology

Trinity College, Dublin

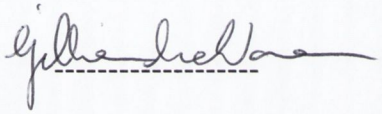
May 1999

TRINITY COLLEGE
- 7 FEB 2000
LIBRARY DUBLIN
Thesis
5286

Declaration

I hereby declare that this thesis has not previously been submitted for a degree at this or any other university and that it is my own work except where duly acknowledged in the text.

I agree that this thesis may be lent or copied at the discretion of the Librarian, Trinity College Dublin, subject to permission granted by Bioresearch Ireland, until the material contained in the thesis is no longer commercially sensitive.



Gillian Mc Namara

Acknowledgements

This thesis is dedicated to my late grandmother, Sheila Cotter, who supported me through many difficult times, both personal and academic.

I would like to thank my supervisor, Professor T.J. Foster for having the idea to carry out this study, and for supporting me for the duration of the project.

Much gratitude also to other members of the academic staff who took an interest, particularly my thesis committee, Prof C.J. Dorman, Prof G.J. Atkins and Dr. Ursula Bond.

Thank you to all my friends and colleagues in the Moyne, whose intellectual input and continuing friendship has helped me enormously over the past few years. I am lucky because there are too many of you to mention individually.

Dr Tom Scott, Dr. Margaret Fitzgerald, Dr. Liz Moran for invaluable help in techniques of monoclonal antibody production.

I am indebted to my husband, Stephen Mulholland for his love and support always, and his interest in my "monoclonal anybodies". Thanks especially for tirelessly entertaining our baby son, Pierce, so often in order to let me complete this thesis. Many thanks to Pierce himself: without your afternoon nap, I could not have written this thesis.

To my in-laws, extended family and non-scientist friends I am grateful for friendship and support also, particularly those that were forced to babysit from time to time so I could write, print or photocopy papers.

For financial support, thanks to Prof. T.J. Foster, Bioresearch Ireland, Forbairt and Trinity Trust.

Summary

Clumping factor (ClfA) of *Staphylococcus aureus* is the major fibrinogen-binding adhesin located on the cell surface of the bacterium. Monoclonal antibodies (mAbs) were produced to two recombinant truncated ClfA proteins, fibrinogen-binding Clf33(221-550) and non-fibrinogen-binding Clf42(332-559). Three mAbs, two against Clf33(221-550) (mAbs 36 and 47) and one against Clf42(332-559) (mAb6) which inhibited the ClfA-fibrinogen interaction were characterized. Each mAb was shown to be specific in recognising ClfA on ELISA. These mAbs inhibited bacterial clumping in soluble fibrinogen and bacterial adherence to solid-phase fibrinogen. Two of these mAbs, 36 and 47, inhibited clumping and adherence synergistically. This antibody combination also blocked *S. aureus* binding to platelet-fibrin clots in vitro. The synergistic mAbs 36 and 47 were also tested in a mouse renal infection virulence model. Using Western immunoblotting of recombinant ClfA truncates, inhibition of mAb binding to Clf41(221-559) with soluble peptides, binding analysis of ClfA site-directed mutant proteins, and reactivity with ClfA-fibrinogen and ClfA-fibrinogen γ-chain 396-412 complexes, these mAbs were shown to define epitopes on ClfA which are functionally important in the fibrinogen-binding mechanism.

Contents

Declaration ii
Acknowledgements iii
Summary iv
List of tables viii
List of figures ix
Quotation x

Chapter 1. Introduction 1
1.1 Biology of staphylococci 1
1.2 Antibiotic resistance of *S. aureus.* 1
1.3 Virulence factors of *S. aureus.* 2
1.3.1 Capsular polysaccharide 2
1.3.2 Secreted toxins 3
1.3.2.1 α-haemolysin 3
1.3.2.2 β-haemolysin 4
1.3.2.3 δ-haemolysin 4
1.3.2.4 Two component toxins 5
1.3.3 Proteins which interact with the immune system 5
1.3.3.1 Protein A 5
1.3.3.2 Toxic shock syndrome toxin 6
1.3.3.3 Enterotoxins 6
1.3.3.4 Epidermolytic toxins 7
1.3.4 Iron acquisition 7
1.3.5 Coagulase 7
1.3.6 Other extracellular fibrinogen-binding proteins 8
1.3.7 Adherence to and survival within endothelial cells 9
1.4 MSCRAMMS of *S. aureus* 9
1.4.1 Wall anchoring mechanism of surface proteins 10
1.4.2 Collagen binding protein 11
1.4.3 Fibronectin binding proteins 12
1.4.4 Binding of *S. aureus* to fibrinogen 13
1.4.4.1 Fibrinogen 13
1.4.4.2 Clumping factor A 14
1.4.4.3 The fibrinogen-binding domain of ClfA. 15
1.4.4.4 ClfA in vivo studies 17
1.4.4.5 Clumping factor B 18

1.4.4.6 SD repeat-containing proteins 19
1.4.5 Non-covalently anchored MSCRAMMS 19
1.4.5.1 Major histocompatibility class II analagous protein 19
1.4.5.2 Elastin binding protein 20
1.4.5.3 Laminin receptor 21
1.4.5.4 Mucin binding protein 21
1.4.5.5 *S. aureus* adherence to other matrix proteins 21
1.5 Global regulatory networks of *S. aureus* 22
1.5.1 The accessory gene regulator system 22
1.5.2 The *sar* regulon 23
1.5.3 In vivo significance of *sar* and *agr* 24
1.6 Aims of project 24

Chapter 2. Materials and methods 25
2.1 *S. aureus* strains 25
2.2 Recombinant protein expression 25
2.3 Nickel-chelate affinity chromatography 25
2.4 Gel filtration chromatography 26
2.5 Polyclonal antibody production 26
2.6 Protein G-sepharose affinity chromatography 26
2.7 Protein concentration determination 27
2.8 Monoclonal antibody production 27
2.9 Indirect ELISA for mAb screening 29
2.10 Isotype analysis 29
2.11 Whole cell dot-blot assay 30
2.12 Monoclonal antibody purification 30
2.13 Soluble clumping assay 31
2.14 Bacterial adherence to solid-phase fibrinogen 31
2.15 Inhibition of fibrinogen-Clf41(221-559) interaction 31
2.16 Plasma clot adherence assay 32
2.17 Mouse infection model 32
2.18 Western immunoblotting of recombinant ClfA proteins 33
2.19 Solid-phase peptide ELISA 34
2.20 Mutant protein ELISA 34
2.21 Soluble peptide inhibition ELISA 34
2.22 Antibody binding to ClfA complexes 34
2.23 Sequence analysis of ClfA 35
2.24 Statistical analysis 35

Chapter 3. Production and identification of anti-ClfA mAbs which inhibit the ClfA-fibrinogen interaction 36
3.1 Introduction 36
3.2 Results 39
3.3 Discussion 43

Chapter 4. Characterization of anti-ClfA mAbs inhibiting the ClfA-fibrinogen interaction 46
4.1 Introduction 46
4.2 Results 51
4.3 Discussion 53

Chapter 5. Adherence of *S. aureus* to platelet-fibrin clots in vitro and virulence models in vivo 58

5.1 Introduction 58
5.2 Results 63
5.3 Discussion 65

Chapter 6. Identification of ClfA epitopes recognised by anti-ClfA mAbs ... 70
6.1 Introduction 70
6.2 Results 75
6.3 Discussion 78

Chapter 7. General discussion 83

References 88

List of Tables

		After page
Table 2.1.	*S. aureus* strains used in this study	25
Table 2.2	ClfA recombinant truncated proteins	25
Table 2.3	ClfA synthetic peptides	34
Table 2.4	ClfA site-directed mutant proteins	34
Table 3.1	Properties of anti-Clf33(221-550) mAbs	40
Table 3.2	Properties of anti-Clf42(332-559) mAbs	41
Table 4.1	Inhibition of *S. aureus* Newman clumping in soluble fibrinogen by individual mAbs	51
Table 4.2	Inhibition of *S. aureus* Newman clumping in soluble fibrinogen by paired combinations of mAbs	51
Table 5.1	Inhibition of *S. aureus* F. Barnett clumping in soluble fibrinogen by mAbs 36 and 47 combined	64
Table 5.2	Data from mouse infection model pilot studies	64
Table 5.3	Data summary for individual mice for infection model	65
Table 6.1	Reactivity of mAbs with ClfA truncated proteins	76
Table 7.1	Data summary for mAbs 6, 36 and 47	83

List of Figures

After page

Figure 1.1. Surface-located proteins of *S. aureus* 5

Figure 1.2 Wall anchoring and sorting of surface-expressed proteins of gram-positive bacteria 10

Figure 1.3 Diagram of the fibrinogen molecule 14

Figure 1.4 Important motifs within ClfA residues 221-559 16

Figure 1.5 SD-repeat containing proteins of *S. aureus* 18

Figure 1.6 Global regulatory networks of *S.aureus* 22

Figure 3.1 Selection of hybridoma cells 36

Figure 3.2 Specificity of anti-ClfA antibodies 42

Figure 3.3 Reactivity of anti-Clf33(221-550) polyclonal IgG with *S. aureus* whole cells 42

Figure 4.1 Inhibition of bacterial attachment to immobilized fibrinogen by anti-Clf33(221-550) polyclonal IgG 52

Figure 4.2 Inhibition of bacterial attachment to immobilized fibrinogen by individual mAbs 52

Figure 4.3 Inhibition of bacterial attachment to immobilized fibrinogen by paired combinations of mAbs 52

Figure 4.4 Inhibition of bacterial attachment to immobilized fibrinogen by cell culture supernatants containing inhibitory mAbs 52

Figure 4.5 Inhibition of fibrinogen binding to immobilized Clf41(221-559)by anti-Clf33(221-550) polyclonal IgG 52

Figure 4.6 Inhibition of fibrinogen binding to immobilized Clf41(221-559)by individual mAbs 53

Figure 4.7 Inhibition of fibrinogen binding to immobilized Clf41(221-559)by paired combinations of mAbs 53

Figure 5.1 Reduction in adherence to platelet-fibrin clots in vitro of a ClfA$^-$ mutant 63

Figure 5.2 Effect of the addition of purified Clf33(221-550) on bacterial adherence to platelet-fibrin clots in vitro 63

Figure 5.3 Inhibition of *S. aureus* adherence to platelet-fibrin clots by anti-Clf33(221-550) polyclonal IgG 63

Figure 5.4 Inhibition of *S. aureus* adherence to platelet-fibrin clots by mAbs 36 and 47 in combination 64

Figure 5.5 Inhibition of *S. aureus* F. Barnett binding to fibrinogen by mAbs 36 and 47 in combination 64

Figure 5.6 Pharmokinetics of mAbs 36 and 47 persistence in the mouse bloodstream 64

Figure 5.7 Bacterial counts in the kidneys of control and passively immunized mice 65

Figure 6.1 Antigenicity profile of ClfA residues 221-559 75

Figure 6.2 Reactivity of anti-Clf33(221-550) polyclonal IgG with immobilized ClfA peptides 76

Figure 6.3 Inhibition of mAbs 36 and 47 binding to Clf41(221-559) by a soluble EF hand (310-321) peptide 76

Figure 6.4 Binding of anti-Clf33(221-550) polyclonal IgG to ClfA mutant proteins 77

Figure 6.5 Binding of mAbs 6, 36, and 47 to ClfA mutant proteins 77

Figure 6.6 Anti-Clf33(221-550) reactivity with Clf41(221-559) complexed in solution with whole fibrinogen or its C-terminal γ-chain 78

Figure 6.7 Binding of mAbs 6, 36 and 47 to Clf41(221-559) complexed in solution with whole fibrinogen or its C-terminal γ-chain 78

Figure 6.8 Effect of the fibrinogen γ-chain and whole fibrinogen on mAb binding to immobilized Clf41(221-559) 78

Figure 6.9 Antigenicity profile for ClfA residues 290-340 79

A cloudy day is no match for a sunny disposition

William Arthur Ward

CHAPTER 1

INTRODUCTION

1.1 Biology of staphylococci

The staphylococci are gram-positive spherical cells usually arranged in grape-like irregular clusters when observed on a Gram stain (Jawetz, 1991). They are members of the normal flora of the skin and mucous membranes of man and animals, and some can cause suppuration, abcesses, and a variety of pyogenic infections and septicaemia. This is as alternative lifestyle, as infection is a rare event caused by organisms that are primarily commensal. The pathogenic staphylococci produce a range of virulence factors. The genus *Staphylococcus* has at least 20 species. *S. aureus* is coagulase-positive and a major pathogen of humans, being responsible for many severe infections. Other pathogenic species for man are *S. lugdunensis*, *S. schlieferi* and *S. haemolyticus*. The coagulase-negative staphylococci are normal human flora. *S. epidermidis* is a major cause of infection of prosthetic devices and *S. saphrophyticus* can cause urinary tract infection in young women. Some other species are important in veterinary medicine. Other invasive diseases caused by staphylococci are endocarditis, septic arthritis, osteomyelitis and myositis. These infections involve the passage of bacteria through blood vessels in order to invade deeper tissue, and this route of infection is primarily a feature of *S. aureus*.

1.2 Antibiotic resistance of *S. aureus*.

Staphylococci have developed resistance to many antimicrobial agents and as a result present difficult therapeutic problems. Multi-drug resistant strains of *S. aureus* are of particular concern. A great proportion of staphylococcal strains causing nosocomial infections are resistant to all antibiotics normally used for treatment except vancomycin (Begley, 1994). Staphylococci are important causes of nosocomial and community-acquired infections (Kayser, 1995). About 20% of bacterial infections are caused by *S. aureus*, an incidence that is exceeded only by that of *Escherichia coli*. In addition, the frequency of staphylococcal disease has increased over the last two decades. Some strains have become multiresistant, to three or more classes of antibiotics. The reason for this is the remarkable genetic versatility of staphylococci, which allow them to adapt quickly to the presence of drugs. In general, the introduction of new antibiotics has resulted sooner or later in the evolution of resistant strains as exemplified by the development of resistance to β-lactam antibiotics. Other antibiotics which *S. aureus* has evolved or acquired

resistance to include aminoglycosides, tetracyclines, fluoroquinolones (Lyon and Skurray, 1987, Paulsen *et. al.,* 1997), and more recently, a low-level resistance to vancomycin has been detected in some clinical isolates (Hiramatsu *et. al.,* 1997).

Relevant to the study of wall-associated proteins, some methicillin-resistant strains of *S. aureus* were found to be defective in the production of major surface antigens such as protein A, ClfA or several other important wall-associated protein known to have a role in virulence (Vaudaux *et. al.,* 1997). These strains contain an intact methicillin resistance determinant (*mec*-element) in their chromosome and were found to be defective in adhesion to immobilized fibrinogen and fibronectin. Western affinity blotting or immunoblotting of wall-associated proteins showed similar levels of fibrinogen or fibronectin binding proteins in MRSA strains compared to a panel of methicillin-sensitive controls. This suggests that the functional expression of the protein is altered by the chromosomal insertion of *mec*, rather than controlling the amount of adhesin produced at the transcriptional or translational level.

1.3 Virulence factors of *S. aureus*

The pathogenesis of *S. aureus* infection is caused by many factors, both extracellular and cell associated (Waldvogel, 1995). *S. aureus* interacts with host-specific targets through a variety of soluble and cell surface components. These multiple mechanisms partly explain the high prevalence of infection with this organism. In addition, this great variety of pathogenic mechanisms, products and toxins probably explains why systemic infections with *S. aureus* still have a high mortality rate, even in the antibiotic era.

1.3.1 Capsular polysaccharide.

The capsule of *S. aureus* consists of polysaccharides and is believed to enhance bacterial virulence by allowing the bacterium to resist phagocytosis and killing by polymorphonuclear leucocytes (Peterson *et. al.,* 1978). Eleven capsular serotypes have been described. Strains expressing type 5 (CP5) or CP8 polysaccharides account for 80 to 85% of clinical blood isolates in septicaemia, providing epidemiological support for their pathogenic significance (Fattom *et. al.,* 1990). CP5 and CP8 are referred to as microcapsules, and their ability to resist phagocytosis is weaker than those of richly encapsulated strains (Lee *et. al.,* 1987). The role played

by the capsule in the virulence of *S. aureus* remains unclear. Using LD_{50} as a clinical outcome parameter, it has been reported that two mutant strains of *S. aureus* devoid of capsular polysaccharide were no less virulent than the parent strain (Albus *et. al.*, 1991). In contrast, a rat model of endocarditis showed that the expression of CP5 and CP8 actually attenuated bacterial virulence possibly by masking adhesins that are critical in the initiation of colonization (Baddour *et. al.*, 1992). In another study, it was found that antibodies to capsular polysaccharide were not protective in the rat endocarditis model (Nemeth and Lee, 1995). It has also been shown that CP5 and CP8 may act as bacterial adhesins and have immunomodulatory effects on endothelial cells and monocytes (Soell *et. al,* 1995). Furthermore, CP5 expression has also been implicated as a virulence determinant in septicaemia and septic arthritis (Nilsson *et. al.*, 1997).

1.3.2 Secreted toxins.

1.3.2.1 α-haemolysin

α-haemolysin is produced by the majority of *S. aureus* strains. It is toxic to a wide variety of mammalian cells and animals, is highly haemolytic for rabbit erythrocytes, and is also dermonecrotic. The mature secreted protein has a molecular mass of 33kDa, and this monomer associates to form cylindrical oligomeric heptamers (Gouaux *et. al.*, 1994). These multisubunit complexes are associated with pore formation in the membrane of the target cell. The crystal structure of this heptamer has been solved, and this has allowed the explanation of the molecular details of the flux of ions and other solutes through the pore (Song *et. al.*, 1996). As α-haemolysin is dermonecrotic and responsible for lethality in a variety of animals, it is likely that this protein is an important virulence factor in human staphylococcal disease. Its effects on the host are multifactorial, mostly as a result of ion imbalances created as a result of pore formation on a variety of cell types (Bhakdi, 1991). Other effects of this toxin include induction of IL-1β secretion by macrophages and disruption of myelin sheaths in rabbit nerve and mouse brain. The major effects of α-toxin relevant to human disease are dermonecrosis, and systemic effects including increased blood coagulation due to an effect on platelets, organ failure due to effects on endothelium and epithelium in lung and possibly cytokine release (Bhakdi and Tranum-Jensen, 1991).

1.3.2.2 β-haemolysin

β-haemolysin is a sphingomyelinase C which is highly haemolytic for sheep erythrocytes but not rabbit red blood cells (Glenny and Stevens, 1935). Also, this toxin is not dermonecrotic in guinea pig skin and not lethal for mice. The haemolytic effect is greatly enhanced by cooling the red blood cells below 10°C after treatment with toxin at 37°C, giving rise to the designation of the hot-cold haemolysin. The *hlb* gene expresses a 35kDa exotoxin (Projan et. al., 1989, Coleman et. al., 1986). The toxin shares 55.7% similarity over a 200-residue domain with a *Bacillus cereus* sphingomyelinase (Yamada *et. al.,* 1988). The role of β-haemolysin in disease is poorly understood. The fact that the toxin is made in abundant quantities by the bacterial cells suggests that it must provide some level of advantage to the organism. It has been shown that β-haemolysin enhances the growth of *S. aureus* in the mouse mammary gland (Foster *et. al.,* 1990). It is strongly associated with bovine isolates, suggesting it is an important virulence factor in mastitis. This toxin is also implicated to have a role in corneal infections. Mutants defective in toxin production lacked the ability to cause scleral oedema in a rabbit keratitis model (O'Callaghan *et. al.,* 1997).

1.3.2.3 δ-haemolysin

δ-haemolysin is a 26-residue peptide which causes damage to the cell membranes of a large selection of mammalian cells. This toxin is made by approximately 97% of *S. aureus* strains, and also may be produced by 50 to 70% of coagulase-negative staphylococci. The *hld* gene encodes δ-haemolysin, giving rise to a transcript of 514 nucleotides (Peng *et. al.,* 1988). The structural gene for the toxin is actually the 3' part of a 45-codon open reading frame that occupies the first 160 nucleotides of a 514 nucleotide transcript, an important part of the accessory gene regulator (*agr*) system that regulates the expression of both cell-associated and secreted virulence factors. The precise role of δ-haemolysin in human disease in unclear. It has broad-spectrum lytic activity for many cell types including erythrocytes, other eukaryotic cells, organelles, spheroplasts and protoplasts (Freer, 1982).

1.3.2.4 Two component toxins

S. aureus can express two distinct types of two-component toxins composed of two independently secreted, non-associated proteins, referred to as class S and class F components (Woodin, 1959, Woodin, 1960). These toxins, γ-haemolysin and the Panton-Valentine leucocidin (PVL), are synergistically toxic (with respect to S and F components) to polymorphonuclear leukocytes and monocytes and macrophages. In addition, γ-haemolysin is capable of lysing erythrocytes from a variety of mammalian species. Almost all *S. aureus* isolates manufacture γ-haemolysin, which is inactivated by sulphonated polymers in agar and therefore not detectable on blood agar plates. However, only 2 to 3% of strains make PVL (Prevost *et. al.,* 1995). Two separate loci give rise to γ-haemolysin and PVL. Within strains capable of making both toxins, three S components (HlgA, HlgC and LukS-PV) and two F components (HlgB and LukF-PV) may be made. Although it is clear that γ-haemolysin and PVL are cytotoxic, their role in disease remains to be elucidated. There is a strong clinical association between strains expressing PVL, which is extremely leucotoxic and severe skin disease (Cribier *et. al.,* 1992, Finck-Barbancon *et. al.,* 1991, Prevost *et. al.,* 1995). γ-haemolysin is also a factor involved in the severity of intraocular infection (Supersac *et. al.,* 1997).

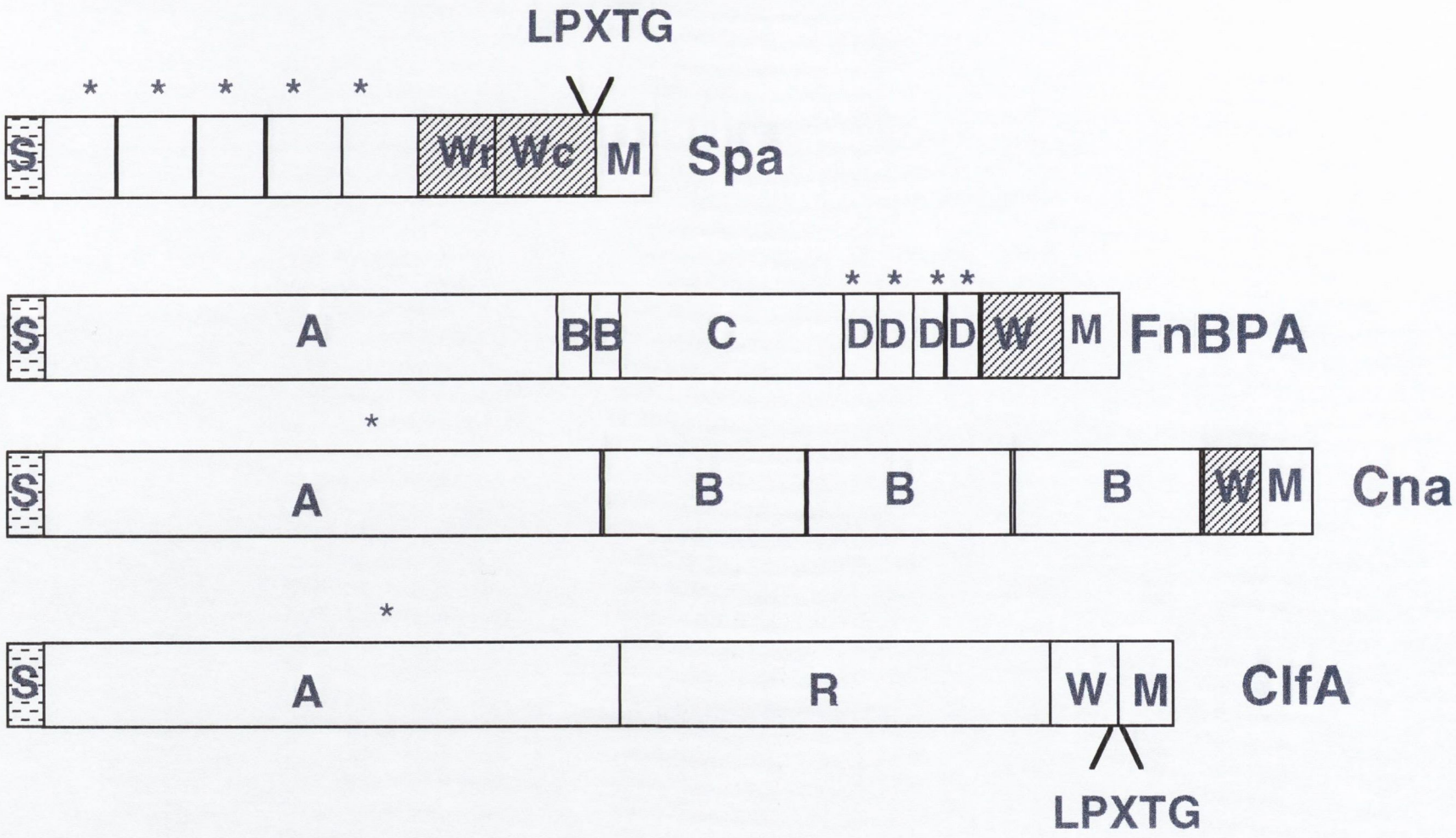
1.3.3 Proteins which interact with the immune system.

1.3.3.1 Protein A.

S. aureus expresses several proteins which interact with the immune system. Protein A (Spa) is a cell wall associated protein which contains a membrane anchor and an LPXTG transpeptidase cleavage site in its C-terminus (Fig.1.1, Guss *et. al.,* 1984). The Spa protein consists of five, or in some isolates four, repeated units of 58 amino acid residues, each of which can bind to the Fc region of IgG (Uhlen *et. al.,* 1984). Protein A interferes with opsonophagocytosis and is an important virulence factor (Patel *et. al.,* 1987).

S. aureus strains with a high Spa content are more resistant to phagocytosis than strains with less of the protein (Peterson *et. al.,* 1977). The antiphagocytic effect is presumably mediated by the binding of Spa to the Fc portion of IgG, thus competing with phagocytic cells for available IgG-Fc sites and thereby resulting in diminished IgG-mediated opsonization. Protein A is also capable of binding to von

Figure 1.1. Organization of wall-associated proteins of *S. aureus*. The principal domains are indicated. S, signal sequence; W, wall-spanning region; M, membrane-spanning domain. Known ligand binding regions are marked by asterisks.


LPXTG
S
Wr
Wc
M
Spa
S
A
B
B
C
D
D
D
D
W
M
FnBPA
S
A
B
B
B
W
M
Cna
S
A
R
W
M
ClfA
LPXTG

Willebrand factor, a protein found where endothelial damage occurs, and may play a role in adherence in the early phase of intravascular infection (Foster and Hook, 1998).

1.3.3.2 Toxic shock syndrome toxin.

Some of the extracellular toxins of *S. aureus* like enterotoxins A-E, epidermolytic toxin A and TSST-1 act as superantigens (Schleivert, 1993) by binding to human and mouse class II major histocompatability complex proteins and stimulating T-cells to proliferate non-specifically (Kim *et. al.,* 1994, Jardetsky *et. al.,* 1994). These superantigens act as important mediators of toxic shock and other acute reactions.

Superantigens are protein molecules which associate with class II major histocompatibility antigens of antigen presenting cells such as macrophages and monocytes through an unusual binding mechanism. This activates T-cell receptors on different subsets of T-lymphocytes. This non-antigen T-cell receptor interaction produces a strong lymphoproliferative response culminating in clonal activation and secretion of a range of cytokines such as interleukin-1 and tumour necrosis factor (Parsonnet, 1989). A situation similar to gram-negative sepsis occurs.

1.3.3.3 Enterotoxins

These proteins have long been associated with a form of gastroenteritis known as staphylococcal food poisoning, but recently have also received attention for causing non-menstrual toxic shock syndrome (Bohach *et. al.,* 1990, Schleivert, 1986). Separate domains of the molecules are known to be responsible for gastroenteritis and superantigenicity (Alber *et. al.,* 1990, Harris *et. al.,* 1993). These molecules have the ability to induce emesis and gastroenteritis upon oral administration to primates. Despite their similarities, antigenic heterogeneity exists and the staphylococcal enterotoxins are currently differentiated into seven major types. These are SEA, SEB, SEC, SED, SEE, SEF, and SEH. Systemic expression during infection results in superantigenic properties. Recently, two new enterotoxin types, G and I, from *S. aureus* have been identified and characterized (Munson *et. al.,* 1998).

1.3.3.4 Epidermolytic toxins A and B

Two serologically distinct epidermolytic toxins have been described, designated epidermolytic toxin A (ETA) and epidermolytic toxin B (ETB). These toxins cause staphylococcal scalded skin syndrome, usually seen in newborns and characterized by intraepidermal separation of layers of the skin at the desmosomes. ETA is chromosomally encoded where ETB is expressed from a large staphylococcal plasmid (Lee *et. al.,* 1987, O'Toole *et. al.,* 1987). Both mature proteins have molecular masses of 27kDa. These toxins are serologically distinct and exhibit only 45% sequence similarity localised to three discrete domains. The molecular mechanism of action of these toxins has recently been discovered (Vath *et. al.,* 1997). The crystal structure of ETA has been solved, and indicates that this toxin belongs to the chymotrypsin-like family of serine proteases. Substitution of the active site serine residue with cysteine abolishes the ability of ETA to produce the characteristic separation of epidermal layers but not its ability to induce T-cell proliferation.

1.3.4 Iron acquisition

Iron availability is of major importance in bacterial pathogenesis, as all bacteria require this essential ion. In response to the low level of available iron in vivo, bacteria induce high-affinity iron uptake systems that consist of low molecular-weight iron chelators, called siderophores, and a number of iron-regulated membrane proteins. Unfortunately, little is known about siderophore production by *S. aureus*. However, a novel siderophore designated aureochelin has been identified recently (Courcol *et. al.,* 1997). Several surface-associated proteins were also shown to be mainly expressed under iron limiting conditions, and these proteins seemed to be recognised by both staphylococcal septicaemia patient sera and polyclonal rabbit serum raised against whole *S. aureus* cells. These proteins could potentially be involved in siderophore uptake.

1.3.5 Coagulase

Most strains of *S. aureus* express coagulase, an extracellular protein that stimulates the conversion of fibrinogen to fibrin to cause the formation of clots in mammalian plasma (Jeljaszewicz *et. al.,* 1983). Coagulase does not have enzymic activity, in that it does not convert prothrombin into thrombin. It binds directly to prothrombin with a 1:1 stoichiometry, and in turn, this complex becomes

proteolytically active and cleaves fibrinogen causing it to polymerize into fibrin (Kawabata *et. al.,* 1985). Eight serotypes of coagulase have been identified (Ushioda *et. al.,* 1981). The primary structures of serotypes I, II, and III have been determined (Kaida *et. al.,* 1989, Kaida *et. al.,* 1987, Phonimdaeng *et. al.,* 1990). The predicted molecular weights of the coagulases vary according to the number of tandem repeats of a 27-amino acid sequence at the C-terminus of the molecule. The prothrombin binding site is located in the N-terminus (Kawabata *et. al.,* 1986, Kawabata *et. al.,* 1985, Phonimdaeng *et. al.,* 1988). The prothrombin-binding regions of the three serotypes contain only 50% identical residues and are probably responsible for the antigenic differences observed by neutralization tests.

Coagulase can also bind to fibrinogen (Boden and Flock, 1989). The binding domain is located in the C-terminus of the molecule as distinct from the prothrombin-binding site (McDevitt *et. al.,* 1992). Despite coagulase being a marker for pathogenic *S. aureus*, little is known about its role in disease. Although several animal models have examined this enzyme, its role in staphylococcal infection is not yet clear. Coa$^-$ mutants do not lack virulence in a rat endocarditis model (Moreillon *et. al.,* 1995) but recently Coa has been implicated in pulmonary infection (Sawai *et. al.,* 1997).

1.3.6 Other extracellular fibrinogen-binding proteins.

Several proteins that are secreted into the extracellular medium by *S. aureus* can bind to fibrinogen. Two of these have coagulase activity. A third, a 19kDa fibrinogen-binding protein designated Fib has been characterized (Boden and Flock, 1994). The *fib* gene encodes a 165-amino acid polypeptide including a 29-residue secretory signal sequence. A sequence of 22 amino acids repeated twice located in the N-terminal portion of Fib shares homology with the C-terminal fibrinogen-binding domain of coagulase, composed of 27 amino acid repeats. The Fib protein was detectable in all *S. aureus* strains with the exception of bovine mastitis strains, probably as a result of proteolytic breakdown (Boden and Flock, 1995). A mutant deficient in Fib results in reduced wound healing (Palma *et al,* 1996) in a rat wound infection model suggesting that Fib may be involved in the pathogenesis of *S. aureus* wound infection and may delay the process of normal healing. Also, a mouse mastitis model has been used to determine if Fib was a protective antigen in this type of

disease (Mamo *et. al.,* 1994). The mice vaccinated with Fib showed reduced rates of infection as compared with controls.

Another novel fibrinogen-binding protein, FbpA has also been identified using a λZap library screened for clones reactive with fibrinogen (Cheung *et. al.,* 1995). Due to its high degree of homology to coagulases already sequenced, it is most likely also a coagulase.

1.3.7 Adherence to and survival within endothelial cells

S. aureus has the ability to colonise and invade endovascular tissue. This could be promoted by the ability of bacteria to adhere to and become internalized by vascular endothelial cells, although a specific *S. aureus* adhesin mediating this interaction was never characterized at the molecular level. However, recent work showed that the dominant pathway for *S. aureus* adherence to human endothelial cells in vitro is mediated by fibronectin-binding protein using fibronectin attached to the endothelial cell surface as a bridge (Peacock *et. al.,* 1999).

Following this initial adherence step, invasion may occur via endothelial cell ingestion. Persistence of the bacteria can occur as they remain viable in this privileged site. Menadione and hemin auxotrophs have been selected by in vitro gentamycin seletion which demonstrated slow growth, reduced coagulase and haemolytic activity, and enhanced intracellular survival (Balwit *et. al.,* 1994). These small colony variants may be adapted especially for evasion of host defences and resistance to antimicrobial therapy. These variants have been associated with persistent and relapsing *S. aureus* infection. Viable *S. aureus* within endothelial cells has been shown to induce apoptosis, and as a result of this, probably contributes to increased tissue damage (Menzies and Kourteva, 1998).

1.4 MSCRAMMS of *S. aureus*

Many pathogenic bacteria bind to extracellular matrix proteins or plasma proteins as a crucial step in the infection process. Fibrin in blood clots, fibronectin in blood clots, fibronectin bridges to host cell surfaces and collagen in cartilage and basement membranes are examples of areas where bacteria may colonise. Adhesins may be involved in staphylococcal infections such as wound, soft tissue and skin infections, endocarditis (see section 5.1), osteomyelitis and septic arthritis. *S. aureus*

is also a major cause of infection associated with prosthetic and intravascular medical devices. Shortly after placement in the body, these devices become coated with host plasma proteins, mainly fibrinogen and fibronectin (Kochwa *et. al.,* 1977). Adhesins expressed on the bacterial cell surface that recognise these matrix proteins can enable the bacteria to colonise and establish foreign body infection (Vaudaux *et. al.,* 1989, Vaudaux *et. al.,* 1993). Initial localised infection can lead to life-threatening diseases such as septicaemia and endocarditis.

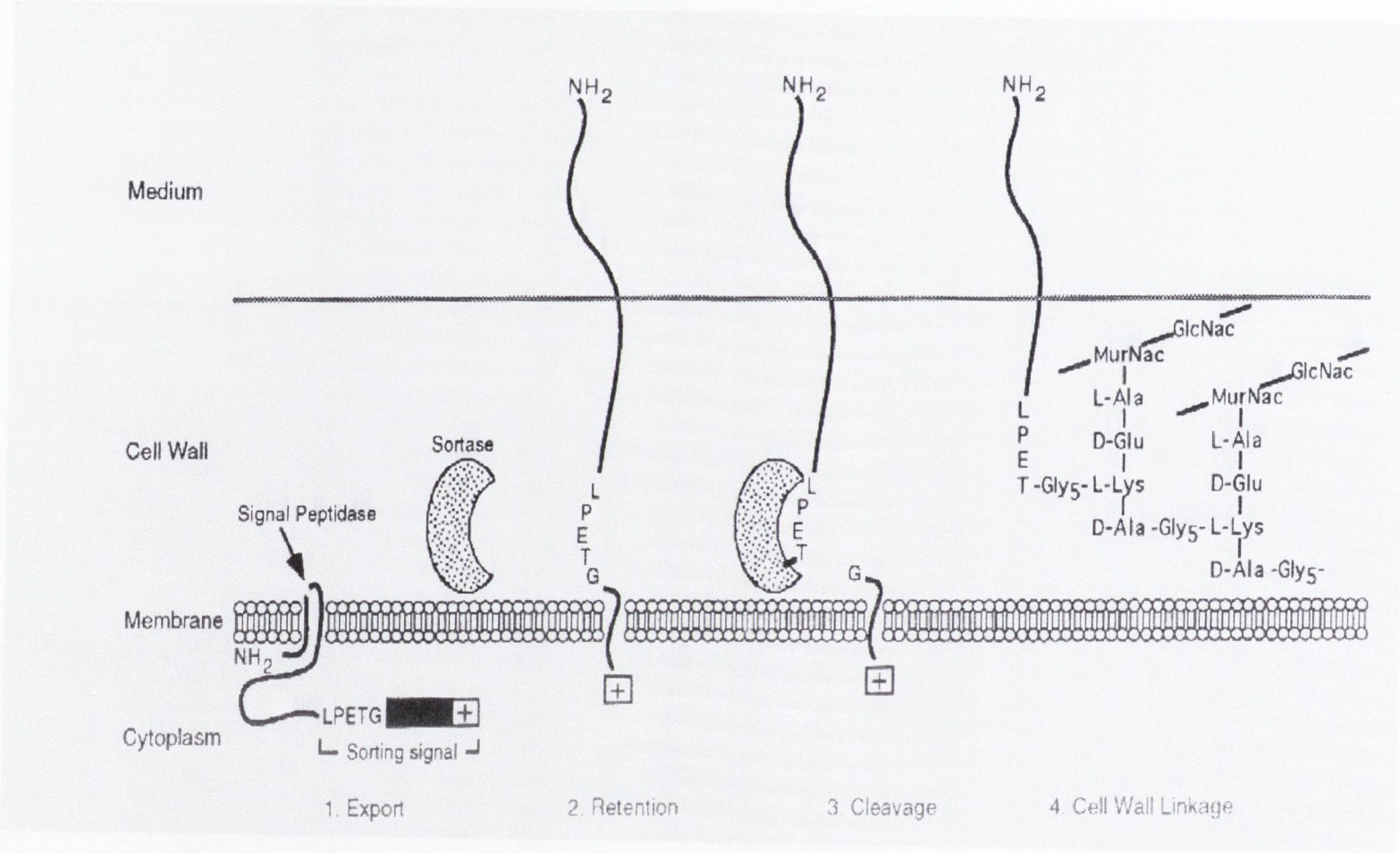
Several proteins have been identified which are expressed on the cell surface of *S. aureus*. These belong to a group of molecules known as MSCRAMMS-microbial surface components recognising adhesive matrix molecules (Patti *et. al.,* 1994). Some of these adhesins have been characterised in molecular detail, and possible roles in virulence identified.

1.4.1 Wall anchoring mechanism of surface-located proteins of gram-positive bacteria

Many staphylococcal surface proteins have certain structural features in common. These features include a secretory signal sequence at the N terminus, positively charged amino acids that extend into the cytoplasm, a hydrophobic membrane-spanning domain, and a cell wall anchoring region, all at the C-terminal of the molecule (Fig. 1.1, Fig.1.5). Ligand-binding domains are usually found in the N-terminus (Patti *et. al.,* 1992, McDevitt *et. al.,* 1994, Guss *et. al.,* 1984). However, the fibronectin-binding proteins A and B are an exception to this as the fibronectin-binding D repeats being close to the wall attachment region (Fig. 1.1, Signas *et. al.,* 1989, Jonsson *et. al.,* 1991).

The cell wall anchoring region of these proteins contains a motif, LPXTG, which is required for cell-wall localisation (Schneewind *et. al.,* 1993). Cleavage occurs between Thr and Gly (Navarre and Schneewind, 1994), and the molecule is covalently linked to the cell wall peptidoglycan by a hypothetical transpeptidase known as sortase (Fig. 1.2, Schneewind *et. al.,* 1995). The carboxyl group of Thr is covalently linked to the carboxyl group of a branch peptide within nascent cross-linked cell wall peptidoglycan (Ton-That *et. al.,* 1997).

Figure 1.2. Sorting and anchoring of wall-associated proteins of gram-positive bacteria.


Medium
Cell Wall
Membrane
Cytoplasm
NH2
NH2
NH2
Sortase
Signal Peptidase
LPETG
LPETG
G
NH2
LPETG
Sorting signal
GlcNac
MurNac
L-Ala
D-Glu
L-Lys
D-Ala -Gly5-
GlcNac
MurNac
L-Ala
D-Glu
L-Lys
D-Ala -Gly5-
1. Export
2. Retention
3. Cleavage
4. Cell Wall Linkage

1.4.2 Collagen binding protein.

The collagen binding MSCRAMM, Cna, has been characterized in detail at the molecular level (Fig. 1.1, Patti *et. al.,* 1993). This protein is thought to be important in the pathogenesis of septic arthritis and osteomyelitis caused by *S. aureus*. It recognises several different types of collagen, including types I, II, III and IV (Patti *et. al.,* 1992).

The Cna protein has an LPXTG motif, a hydrophobic membrane-spanning domain and a positively charged C-terminal tail (Patti *et. al.,* 1992). Initially, the ligand-binding domain of this 135kDa adhesin was localised using deletion mutagenesis (Patti *et. al.,* 1993). The collagen-binding domain was localised to a 168 amino acid long segment (residues 151-318) within the N-terminal portion of the protein. Short truncations in the terminal flanking regions of the 151-318 recombinant protein resulted in the loss of collagen-binding activity.

The collagen-binding site has been identified within the adhesin in a region between amino acids Asp209 and Tyr233 (Patti *et. al.,* 1995). When overlapping synthetic peptides mimicking segments of the ligand-binding domain of the adhesin were tested for their ability to neutralise the inhibitory activity of a blocking polyclonal antibody, one peptide was found to be active. A site-directed double mutant recombinant protein that was defective in residues N232A and Y233A exhibited dramatic changes in collagen-binding activity as compared to the wild-type recombinant protein.

The crystal structure of the ligand-binding domain has been determined (Symersky *et. al.,* 1997). This 19kDa subdomain has a "jelly-roll" fold consisting of two anti-parallel β-sheets and two short α-helices. A "trench" crosses one of these β-sheets, and molecular modelling showed that this trench can accomodate a collagen triple helix. Site-directed mutations designed to interfere with the walls of the putative binding site resulted in a mutant protein with reduced affinity for collagen.

Using Cna coated beads, the presence of Cna was found to be sufficient for the in vitro adherence of bacteria to cartilage (Switalski *et. al.,* 1993). Polyclonal antiserum raised against Cna blocked *S. aureus* adherence to both purified collagen and cartilage. The ability of Cna to mediate bacterial attachment makes it an important virulence factor in the pathogenesis of septic arthritis. A Cna^- strain was found to be significantly less virulent in a mouse septic arthritis model than the wild-

type strain (Patti *et. al.,* 1994). This protein may also have a role in the pathogenesis of infective endocarditis (Hienz *et. al.,* 1996). Although Cna did not seem to be involved in the initial colonisation of a traumatized heart valve, a strain expressing Cna proliferated more than an isogenic Cna$^-$ mutant.

This adhesin may also be a potential vaccine antigen, as mice vaccinated with a recombinant fragment of Cna were protected against sepsis-induced death (Nilsson *et. al.,* 1998). Protection by passive immunization was also observed. In vitro experiments indicated that antibodies against this adhesin promoted phagocytic uptake and enhanced intracellular killing.

1.4.3 Fibronectin-binding proteins.

S. aureus is known to express two fibronectin receptors on the cell surface, FnBPA and FnBPB (Fig. 1.1, Signas *et. al.,* 1989, Jonsson *et. al.,* 1991). The genes encoding these proteins are linked on the staphylococcal chromosome separated by 682bp. These proteins have all the features associated with surface proteins from gram-positive bacteria (Schneewind *et. al.,* 1993). Analysis of recombinant fragments of FnBPA localized the fibronectin-binding domain to the D-repeats, 38-residue repeated domains located directly N-terminal of the LPXTG motif (Signas *et. al.,* 1989). Each D-repeat is capable of binding to fibronectin, as synthetic peptides mimicking each individual repeat can reduce *S. aureus* binding to fibronectin (Rampyari *et. al.,* 1990). The D-repeats recognise repeated domains on the fibronectin molecule, the type I modules in the N-terminal portion of the protein. Mutations in any of these crucial type I modules reduced the affinity of the recombinant molecules for FnBPA (Sottile *et. al.,* 1991). This demonstrates that the multiple nature of the interaction between these two proteins is important.

Site-specific mutants in FnBPA and FnBPB of *S. aureus* 8325-4 showed reduced abilities to attach to fibronectin-coated coverslips (Greene *et. al.,* 1995). The single mutants had a slightly reduced binding ability, while the double mutant could did not demonstrate any fibronectin-binding activity. This shows that both proteins are important for the adherence of this strain to fibronectin-coated surfaces. This double mutant was also deficient in binding to ex vivo coverslips. However, not all strains possess two *fnb* genes. Southern blotting analysis showed that some phage group I and II strains only have one *fnb* gene (Greene *et. al.,* 1996).

These proteins have been shown to have a role in adherence to ex vivo biomaterial that has been in long term contact with host proteins (Vaudaux *et. al.*, 1993, Vaudaux *et. al.*, 1995) indicating their involvement in device-related infection. FnBPA and FnBPB may also be involved in the pathogenesis of infective endocarditis as a low-fibronectin binding mutant was found to have reduced adherence to traumatized heart valves in a rat model of endocarditis (Kuypers and Proctor, 1989). However, a more recent study found that a strain lacking FnBPA and FnBPB showed the same level of infectivity, indicating that other factors on the cell surface may compensate for the lack of fibronectin binding. The expression of the fibronectin-binding proteins is maximal during exponential growth, and disappears rapidly but only in some strains, on entry into stationary phase. This loss has been shown to occur as a result of the activity of V8 protease (McGavin *et. al.*, 1997).

In the absence of fibronectin, the D repeats exist in a loose unordered conformation (House-Pompeo *et. al.*, 1996). Monoclonal antibodies have also been isolated which caused these repeats to become more ordered, and actually enhanced the binding of fibronectin to a recombinant protein containing the ligand-binding domain (Speziale *et. al.*, 1996). These are called ligand-induced binding sites (LIBS).

1.4.4 Binding of *S. aureus* to fibrinogen

1.4.4.1 Fibrinogen

Fibrinogen comprises 2 to 3% of plasma protein (Voet and Voet, 1990). A molecule of fibrinogen consists of three pairs of non-identical but homologous polypeptide chains, Aα (610 residues), Bβ (461 residues) and γ (411 residues). This complex also contains two pairs of N-linked oligosaccharides of approximately 2.5kD each. A and B represent the 16- and 14-residue fibrinopeptides that thrombin cleaves from fibrinogen so that a fibrin monomer is designated $\alpha_2\beta_2\gamma_2$. The reaction forming a blood clot involves the cleavage of the fibrinopeptides A and B, and subsequent association of fibrinogen monomers to form fibrin.

Combined electron microscopic and low resolution X-ray crystallographic studies indicate that fibrinogen is a twofold symmetrical elongated molecule about 45nm in length. It has two nodules at each end and one in the middle. Its six polypeptide chains are joined by 17 disulphide bonds, 7 within each half of the dimer and 3 linking these two protomers. Structural indications together with sequence-

based conformation predictions suggest that the central region of each protomer consists mainly of a three stranded coiled coil of α-helices in which the α, β and γ chains each contribute a strand (Doolittle, 1984). Thrombin specifically cleaves the Arginine-X peptide bond (where X is Glycine in most species) joining each fibrinopeptide to fibrinogen. Fibrin then aggregates spontaneously to form fibres that electron micrographs indicate have a banded structure that repeats every 22.5nm (Fig. 1.3).

Fibrinogen is prevented from associating non-specifically with itself by charge-charge repulsion. The loss of the fibrinopeptides, which are highly negatively charged, have a marked change on the overall charge of the complex. The attraction after fibrinopeptide cleavage between fibrin's central and end segments promotes its specific association.

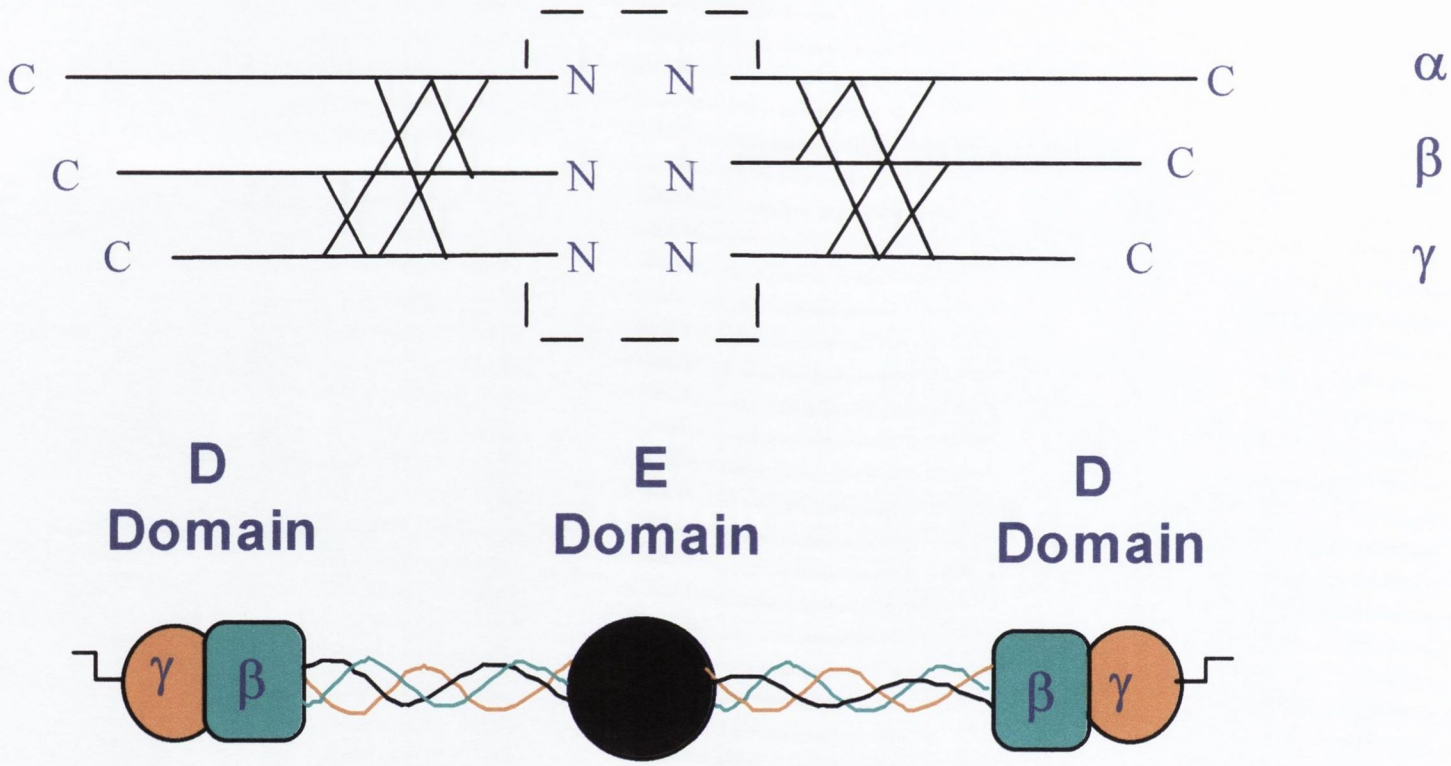
1.4.4.2 Clumping Factor A

It has been known since the beginning of the century that *S. aureus* can form clumps in plasma (Much, 1908). This organism can also coagulate plasma, a phenomenon due to a predominantly extracellular protein, coagulase, which binds to prothrombin with 1:1 stoichiometry and activates the clotting cascade (see section 1.3.5), (Kawabata *et. al.,* 1985). It was originally thought that clumping was due to a cell-bound form of coagulase. As early as 1954, Duthie suggested that clumping factor and coagulase were in fact separate entities (Duthie, 1954). There were several reports of purification of clumping factor (Chatwal *et. al.,* 1987, Switalski, 1976) but none that characterized the protein very well.

It has been previously demonstrated that coagulase can bind to fibrinogen as well as prothrombin, and also that a fraction of coagulase is bound firmly to the cell wall (Boden and Flock, 1989). These observations were confirmed (McDevitt *et. al.,* 1992) and the fibrinogen-binding site was localised to the C-terminal 177 amino acids of the molecule. This site is distinct from the prothrombin-binding domain located in the N-terminus. Also in this study, a mutation was constructed by allelic replacement in the *coa* gene of *S. aureus* strain Newman, which abolished coagulase activity. This mutant retained the ability to clump in soluble fibrinogen and to adhere to fibrinogen-coated coverslips.

Mutants defective in clumping were subsequently isolated by Tn*917* mutagenesis (McDevitt *et. al.,* 1994). These mutants failed to clump in soluble

Figure 1.3. Representation of the fibrinogen molecule.


C
C
C
N
N
N
N
N
N
C
C
C
α
β
γ
D
Domain
E
Domain
D
Domain
γ
β
β
γ

fibrinogen and to adhere to solid-phase fibrinogen. The gene was cloned and sequenced, and predicted to express a 92kDa or 896 residue protein. A single copy of the gene reintroduced into the chromosome of the mutants fully complemented the mutants. This gene was introduced into strain 8325-4, a weakly clumping strain, on a shuttle plasmid and allowed this strain to clump and significantly enhanced its adherence. The ClfA protein (Fig. 1.1) has approximately 20% similarity to the fibronectin-binding proteins A and B in region A (Jonsson *et. al.,* 1991, Signas *et. al.,* 1989) and was also recently found to have less extensive homology to an integrin-like protein expressed on the surface of *Candida albicans* (Gale *et. al.,* 1996).

There are several negatively charged residues at the extreme C-terminus of ClfA which are presumed to act as a stop-transfer signal during secretion across the cell membrane. A small hydrophobic region from A905 to F923 probably spans the cytoplasmic membrane. The C-terminus of ClfA also contains a consensus motif, LPDTG, which is required for cell-wall localisation in Gram-positive bacteria (Schneewind *et. al.,* 1993). ClfA lacks a Pro-Gly rich region present in most surface-associated proteins and assumed to span the cell wall. It contains a novel Asp-Ser dipeptide repeat region, of varying length depending on strain (McDevitt *et. al.,* 1995a). This unusual repeat domain, region R, is required for the surface display of the fibrinogen-binding region A (Hartford *et. al.,* 1997). To determine the minimum length of region R required for wild-type levels of ClfA expression, variants with truncated region R domains were constructed. It was found that greater than 72 residues were required to allow region A to span the staphylococcal cell wall sufficiently to have full functional fibrinogen-binding activity. A sequence of 39 residues at the N-terminus has all the features of a typical bacterial signal sequence, including a positively charged N-terminus, a hydrophobic core and a polar cleavage region.

1.4.4.3 The fibrinogen-binding domain of ClfA

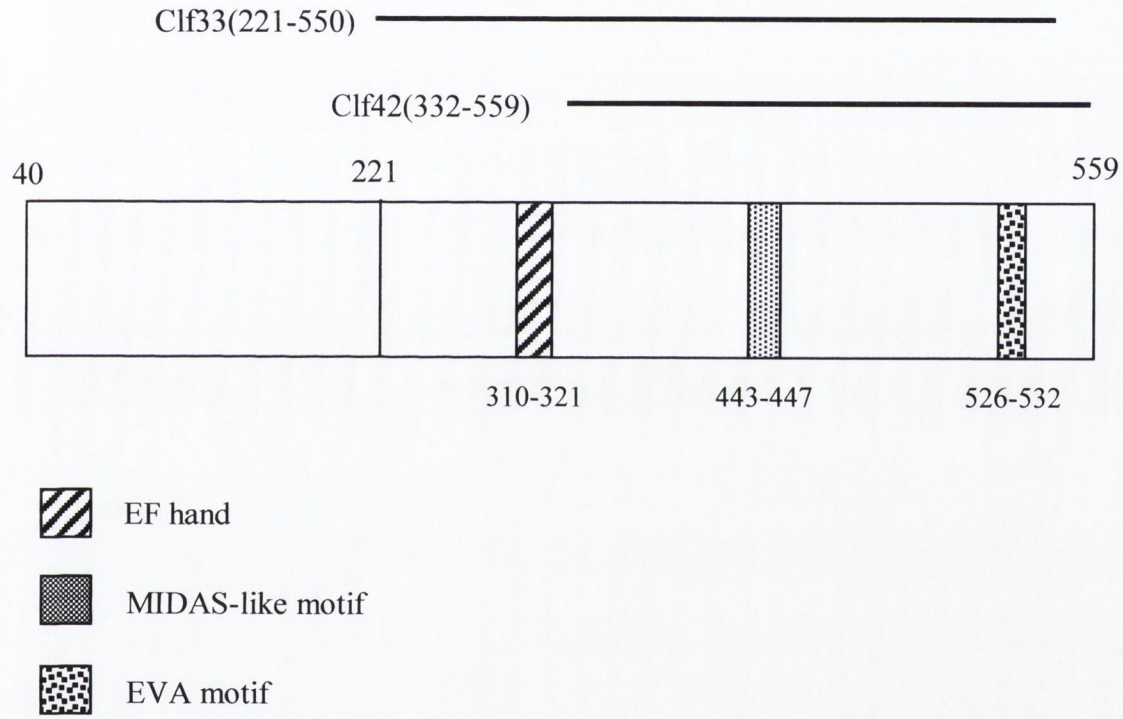
The N-terminal portion of the protein, region A, contains the fibrinogen-binding domain. This domain was further localised to between residues 221-550 within region A (McDevitt *et. al.,* 1995b). This was achieved by analysis of fibrinogen binding by recombinant truncates of region A. A similar approach was used to locate the collagen-binding domain of Cna (Patti *et. al.,* 1993). Polyclonal antiserum raised against region A inhibited both clumping and adherence to solid-phase fibrinogen.

Immunofluorescense microscopy using this antiserum showed that region A is exposed on the cell surface. Region A coupled to polystyrene beads caused clumping in soluble fibrinogen demonstrating that this protein alone is sufficient for the clumping phenotype. The Clf33 recombinant truncate (residues 221-550) is the minimum which still retains fibrinogen-binding activity. Another truncate, the Clf42 protein (residues 332-550), which has no fibrinogen-binding activity, can neutralise the effect of blocking polyclonal antiserum.

It has been shown that a synthetic peptide corresponding to the C-terminal 17 residues of the γ-chain of fibrinogen inhibited clumping of *S. aureus* in soluble fibrinogen (Hawiger *et. al.*, 1982). It has also been demonstrated that purified recombinant ClfA bound to these same residues of fibrinogen. A synthetic peptide corresponding to the C-terminal γ-chain of fibrinogen inhibited binding of fibrinogen to ClfA (McDevitt *et. al.*, 1997). This region of fibrinogen also interacts with the α-subunit of platelet integrin $\alpha_{IIb}\beta_3$ (D'Souza *et. al.*, 1990). The γ-chain binding site has been mapped to a region of the α_{IIb} chain that contains a short sequence with homology to a motif found in eukaryotic Ca^{2+} binding proteins, an EF hand. This motif contains 13 amino acids, where positions 1, 3, 5, 7 and 12 are occupied by oxygenated residues. A solvent molecule is associated with residue 9 by a hydrogen bond (Barioch, 1991). These residues form a sphere of coordination for Ca^{2+} and are flanked by α-helices. Co-operative binding of multiple cations is common, and more than one EF hand motif can be found within the same protein. Chemical cross-linking studies showed that an EF-hand like sequence was responsible for fibrinogen binding (D'Souza *et. al.*, 1990). A peptide corresponding to the EF hand bound to fibrinogen in a Ca^{2+}-dependent manner (D'Souza *et. al.*, 1991). This evidence suggested that EF hands within other molecules, such as ClfA, could interact with the C-terminal γ-chain of fibrinogen. Further characterisation of $\alpha_{IIb}\beta_3$ shows that there are two kinds of Ca^{2+} binding sites (Hu *et. al.*, 1996). High affinity sites promote ligand binding while low affinity sites prevent protein-protein interactions.

EF hand-like motifs do indeed occur within 221-559 of ClfA (O'Connell *et. al.*, 1998). The best potential site was found at residues 310-321 within this minimum fibrinogen-binding domain (Fig. 1.4). Deletion mutagenesis and site-directed mutagenesis of this motif in recombinant truncated ClfA proteins 40-559 and 221-559

Figure 1.4. Motifs of significance in ClfA region A which may be important in the ClfA-fibrinogen interaction.


Clf33(221-550)
Clf42(332-559)
40
221
559
310-321
443-447
526-532
EF hand
MIDAS-like motif
EVA motif

caused a significant reduction in affinity of these proteins for whole fibrinogen and the fibrinogen γ-chain peptide. The divalent cations Ca^{2+} and Mn^{2+} inhibited the interaction of the peptide to recombinant region A and also the adherence of *S. aureus* cells to solid-phase fibrinogen. Mg^{2+} or Na^{2+} at similar concentrations had no effect on these interactions. Far-UV spectroscopy indicated that Ca^{2+} and Mn^{2+} induced changes in secondary structure of 40-559 and 221-559. These results suggest that Ca^{2+} binds to an inhibitory site or sites within ClfA region A and causes a conformational change. This altered state is not compatible with ClfA binding to the C-terminal γ-chain of fibrinogen. Mutagenesis studies seem to indicate that this Ca^{2+}-dependent inhibitory site is situated at residues 310-321 within the EF hand motif (Fig. 1.4).

The leucocyte integrin $\alpha_M\beta_2$ recognises a part of the fibrinogen γ-chain (Altieri *et. al.,* 1993). Ligand binding by this integrin involves a 200-residue insertion of an A (or I) domain in the α chain. This domain contains a novel cation-binding metal ion-dependent adhesion site (MIDAS) motif (Lee *et. al.,* 1995). The integrity of the MIDAS motif is essential for integrin-ligand interaction. ClfA has a MIDAS-like motif at residues 443-447 (Fig. 1.4) which may be involved in binding to the γ-chain of fibrinogen, although there is no direct evidence to support this. However, site-directed mutants in this motif had a reduced ability to bind to fibrinogen, but showed the same level of interaction as the wild-type protein with the fibrinogen C-terminal γ-chain peptide (O'Connell, Personal communication)

A set of site-directed mutations in the C-terminus of ClfA region A at residues 526-532 showed that this area may have a role in the ClfA-fibrinogen interaction (Fig.1.4). When certain of these residues were mutated, there was a marked reduction in the fibrinogen-binding ability of the purified recombinant proteins. This was also shown when the mutations were transferred into a ClfA molecule expressed on the bacterial cell surface (Hartford, Personal communication).

1.4.4.4 ClfA in vivo studies

The role of host proteins in promoting staphylococcal adherence to biomaterials has been investigated using an ex vivo canine arteriovenous shunt model (Vaudaux *et. al.,* 1995). The results of this study suggested that fibrinogen is the foremost adhesion-promoting protein in short term blood-biomaterial interaction. It was

previously thought that fibrinogen was not as important as fibronectin for promoting staphylococcal attachment. This was based on a study performed using catheters removed from patients after being in vivo for several days (Vaudaux *et. al.,* 1993). It seems that fibrinogen is more important in promoting the initial attachment of the organism, where fibronectin is probably more important in the long term due to the rapid degradation of fibrinogen in vivo.

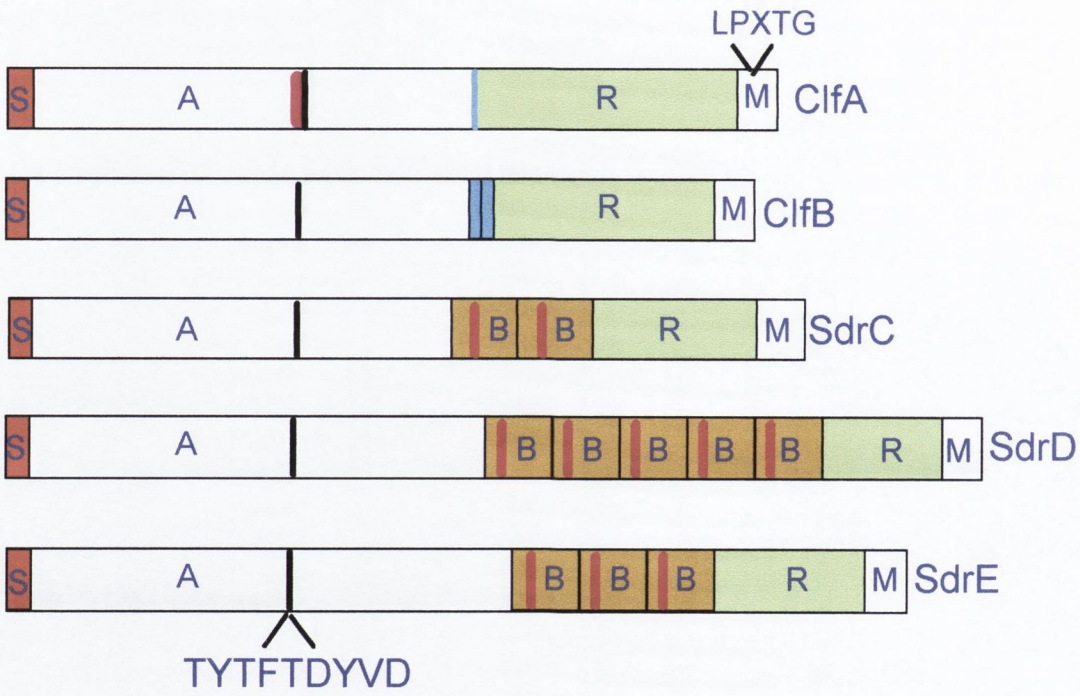
The role of *S. aureus* coagulase and clumping factor in a rat endocarditis model has been examined (Moreillon *et. al.,* 1995). Coagulase-defective mutants were not affected in their infectivity and were not altered in their ability to adhere to platelet-fibrin clots in vitro. Clumping factor negative mutants produced 50% less endocarditis than the wild-type strain Newman, depending on inoculum size. This mutant was also significantly less adherent to platelet-fibrin clots. Complementation of the clumping factor mutant with a copy of the *clfA* gene restored adherence in vitro and infectivity in the endocarditis model. Thus, ClfA is an important virulence factor in this model.

1.4.4.5 Clumping factor B

Southern blotting analysis showed that there were several other loci present in the staphylococcal genome that contained this dipeptide repeat sequence (McDevitt *et. al.,* 1994). The genes encoding these novel surface-associated proteins have been isolated (Fig. 1.5, Ni Eidhin *et. al.,* 1998, Josefsson *et. al.,* 1998).

The first of these loci to be characterized at a molecular level encodes a fibrinogen-binding protein known as clumping factor B (ClfB)(Ni Eidhin *et. al.,* 1998). This protein has considerable sequence identity with ClfA in the signal sequence and wall attachment regions. However, the A regions only share 26% identity. ClfB was found to bind the α- and β-chains of fibrinogen on Western affinity blots, where ClfA binds only to the γ-chain. The *clfB* gene product is a 124kDa protein. This protein was found to be expressed only in early exponential phase of growth. It cannot be detected in late exponential or stationary phase cells. Using an isogenic *clfB* mutant, both alone and in combination with a mutation in the *clfA* gene, ClfB was shown to increase clumping of exponential phase cells in soluble fibrinogen, adherence of exponential phase bacteria to solid-phase fibrinogen in vitro

Figure 1.5. Organization of the SD-repeat containing proteins of *S. aureus*.


LPXTG
S
A
R
M
ClfA
S
A
R
M
ClfB
S
A
B
B
R
M
SdrC
S
A
B
B
B
B
B
R
M
SdrD
S
A
B
B
B
R
M
SdrE
TYTFTDYVD

and bacterial adherence to ex vivo human haemodialysis tubing. These findings imply that ClfB could be involved in the pathogenicity of foreign device related infections.

In wild-type exponential phase *S. aureus* Newman cultures, ClfB activity was masked by ClfA activity. ClfB was not involved in binding to fibrinogen when cells were in stationary phase. Similar to ClfA, millimolar concentrations of Ca^{2+} and Mn^{2+} inhibited ClfB mediated adherence to solid-phase fibrinogen. This suggests that ClfB fibrinogen binding is also regulated by a low affinity inhibitory divalent cation-binding site.

1.4.4.6 SD-repeat containing proteins.

Three new SD repeat-containing genes besides ClfA and ClfB have recently been identified in *S. aureus* (Josefsson *et. al.,* 1998, Fig. 1.5). These three genes are closely linked on the staphylococcal chromosome and are present together in tandem. The A regions of these proteins only exhibit 20-30% amino acid identity with each other. However, one conserved motif is present in all five members of this family of proteins, TYTFTDYVD. The Sdr (SD repeat-containing) proteins also contain two to five extra 110-113 residue domains, (B-motifs) found between regions A and R within the proteins. Each B repeat contains an EF hand sequence similar to that found in residues 310-321 of ClfA (O'Connell *et. al.,* 1998). There is also evidence to suggest that the EF hands within the B repeats of SdrD are functional, as the conformation of a recombinant protein composed of the five B-repeats of this protein was found to be Ca^{2+}-dependent. The R-domains were found to contain 132-170 SD repeat units. Other conserved areas between the five proteins in this family occurred at the N-terminal signal sequence and the conserved wall anchor region. Although this *sdr* locus was present in all of 31 *S. aureus* strains analysed by Southern hybridization, the function and the role of these proteins in the pathogenicity of this organism has yet to be elucidated.

1.4.10 Non-covalently anchored MSCRAMMS

1.4.10.1 Major histocompatability class II analagous protein

In an attempt to identify a receptor for bone sialoprotein, a staphylococcal surface protein capable of identifying several extracellular matrix glycoproteins was purified (Homonylo McGavin *et. al.,* 1993). This protein was later characterised by

cloning and sequencing of the gene (Jonsson *et. al.,* 1996). The deduced protein consists predominantly of six repeated domains of 110 amino acids. Each of these contains a subdomain of 31 residues that has strong homology with a segment in the peptide binding groove of the β chain of the major histocompatibility complex (MHC) class II family of molecules. This MHC class II analogous protein (MAP) is capable of binding to several extracellular matrix proteins, including fibronectin, fibrinogen, vitronectin, bone sialoprotein, and thrombospondin. The purified recombinant bacterial protein has also been shown to specifically recognise a 15-amino acid synthetic peptide derived from vitronectin. This protein may recognise carbohydrate residues present on host matrix glycoproteins. However, this protein does not have an LPXTG motif and it does not seem to be covalently linked to the cell wall as it can be released from cells by treatment with LiCl. No surface-localization experiments have been performed in order to prove that this molecule is a cell wall-associated adhesin.

1.4.10.2 Elastin binding protein.

Binding of elastin by *S. aureus* may contribute to the infection of sites in the body which contain this protein such as the skin, lungs and blood vessels. *S. aureus* can bind to the tropoelastin monomeric form which makes up mature elastin fibres and also elastin degradation peptides (Park *et. al.,* 1991). The protein on the bacterial cell mediating this interaction with elastin had previously been identified as a 25kDa antigen, EbpS (Park *et. al.,* 1996). Similar to MAP, this protein does not contain a classical C-terminal wall attachment region seen in other surface expressed antigens of gram-positive bacteria. There is evidence to suggest that the 25kDa form present on the cell surface is processed post-translationally from a 44kDa precursor, although the mechanism for the processing and cell wall localization of this protein has not yet been identified.

The gene expressing EbpS has recently been cloned and sequenced, and found to be 487 amino acids in length (Roche, Personal communication). The ligand-binding domain is localized in the N-terminus within residues 14-34. Full length recombinant EbpS migrated at an apparent molecular mass of 80kDa. The protein was found to lack an

N-terminal signal peptide and an LPXTG motif, indicating a novel mechanism for secretion and wall attachment. Three large hydrophobic domains were found at the

C-terminus, which may have a role in secretion and correct surface expression of the ligand-binding domain. Also, a 50-residue region at the extreme C-terminus was homologous to a repeat domain found in a subset of bacterial cell wall hydrolases. An *ebpS* null mutant has been constructed and will be used for in vitro adherence assays and virulence studies.

1.4.10.3 Laminin receptor

A characteristic feature of staphylococcal infection is bloodstream invasion and widespread metastatic abcess formation. It has been shown that *S. aureus* is capable of binding to the basement membrane protein laminin which may explain its ability to cross the vascular basement membrane and promote infection (Lopes *et. al.,* 1985). Normal and neoplastic mammalian cells also use this mechanism in order to extravasate. The estimated molecular mass of this receptor was 50kDa, and about 100 binding sites per cell were found. Also, a mAb that could block the binding activity of this receptor was also capable of recognising other laminin-binding proteins, indicating that functionally important epitopes were conserved (Vincentini *et. al.,* 1997). However, the bacterial protein has never been identified and characterized at the molecular level.

1.4.10.4 Mucin binding protein.

20-30% of normal adults are colonised permanently with *S. aureus* in the anterior nares. Colonisation of human nasal mucosa with *S. aureus* may be the initiating factor for subsequent systemic infection (Shuter *et. al.,* 1996). This may occur through binding of bacteria to human nasal mucin. This occurs partly via specific adhesin-receptor interactions involving bacterial wall-associated proteins and the carbohydrate component of mucin. *S. aureus* binding to mucin in the nasal cavity of humans may be critical for colonisation of the nasopharyngeal mucosa.

1.4.11 *S. aureus* adherence to other matrix proteins.

S. aureus can also bind to other tissue and matrix proteins such as bone sialoprotein (Ryden *et. al.,* 1989), vitronectin (Paulsson et. al., 1992), thrombospondin (Herrmann *et. al.,* 1991), von Willebrand factor and osteopontin (Foster and Hook, 1998). However these interactions are poorly characterised at the

molecular level. The interaction of *S. aureus* with thrombospondin, the major platelet protein, may be important in the pathogenesis of foreign body infections.

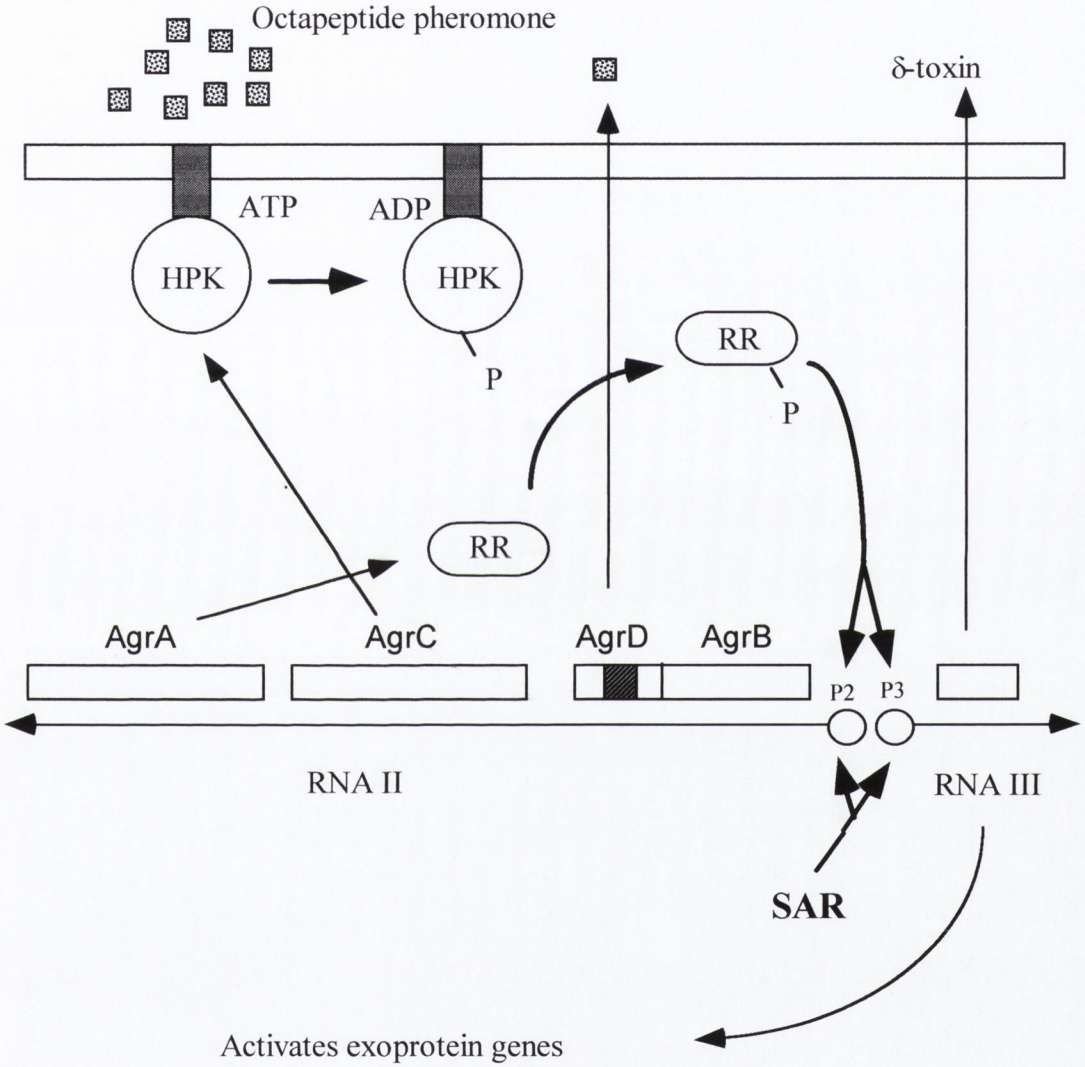
1.5 Global regulatory networks of *S. aureus*

1.5.1 The accessory gene regulator system

The expression of many *S. aureus* secreted proteins is regulated by the *agr* system. These *agr*-regulated genes include the cytolytic toxins α-, β-, γ- and δ-haemolysins, enterotoxins, toxic shock syndrome toxin-1, PV-leucocidin, protein A, coagulase and others (Dorman, 1994). The expression of genes under the control of the *agr* locus is differentially regulated such that those expressed during stationary phase, predominantly secreted proteins, are activated by *agr* while genes expressed during exponential growth are repressed by *agr*. This sequential expression of genes may have clinical importance. (Fig. 1.6)

A Tn*551* insertion mutation in *agr* decreased expression of α-haemolysin, β-haemolysin, δ-haemolysin, TSST-1, enterotoxin B, epidermolytic toxins A and B, leucocidin, staphylokinase, nuclease, serine protease, metalloprotease and acid phosphatase and increased expression of protein A and coagulase (Gaskill and Khan, 1988, Janzon *et. al.,* 1990, O'Toole and Foster, 1986, O'Toole and Foster, 1987, Recsei *et. al.,* 1985). Cloning and sequencing has shown that this Tn*551* insertion is within an open reading frame whose protein (AgrA) is homologous to the response regulators of the histidine protein kinase/response regulator two component family (Peng *et. al.,* 1988). This gene is within a polycistronic operon where the genes *agrB*, *agrC* and *agrD* are also present (Janzon *et. al.,* 1990, Vandenesch *et. al.,* 1991). The *agr* locus also includes the *hld* gene (encoding δ-haemolysin) which is transcribed divergently from *agr* on the opposite DNA strand. The transcriptional organisation of *agr* is complex. *agrA* is transcribed from its own weak constitutive promoter, P1, as a monocistronic message known as RNAI. *agrA* and *agrBCD* are transcribed as a polycistronic message (RNAII) from the P2 promoter. A third promoter P3 transcribes *hld* on the opposite strand. AgrC has homology with the histidine protein kinase family of sensor proteins and is believed to phosphorylate in response to environmental stimuli. The phosphate group is then transferred to AgrA (Janzon *et. al.,* 1990). The AgrC protein shows regions of hydrophobicity indicating

Figure 1.6. Known pathways for coordinate control of gene expression in *S. aureus.*



Activates exoprotein genes
Represses surface protein genes

that it may be membrane-associated (Vandenesch *et. al.,* 1991). AgrA is a positive regulator of the *agr* locus itself. Activation of AgrA results in an increase in *hla* transcription showing that *hla* and *agr* are co-regulated at the transcriptional level (Janzon *et. al.,* 1990).

The *hld* message is a 517 nucleotide transcript called RNAIII and is translated to give a secreted 26 residue peptide which forms channels in lipid bilayers causing lysis of cells or organelles as a result (Freer, 1988). The *hld* transcript seems to regulate *agr*-dependent genes. The initial stimulus for switching on *agr* is cell density (Balaban and Novick, 1995, Ji *et. al.,* 1995). As cells grow exponentially from a low cell density they probably express a low basal level of the agr P2 operon. The AgrD gene product seems to be cleaved proteolytically by AgrB and this causes the secretion of an octapeptide pheromone into the medium. When this peptide reaches a certain concentration, it is thought to bind to the domain of AgrC that is exposed on the outer face of the cell membrane. This activates the protein kinase activity of AgrC and the response regulator AgrA, and the P2 operon transcription is stimulated.

1.5.2 The *sar* regulon

Another regulatory locus, *sar*, is also involved in the pleiotropic expression of both extracellular and cell wall proteins (Cheung and Ying, 1994). This locus has been found to regulate protein A synthesis at the transcriptional level in addition to *agr* (Cheung *et. al.,* 1997). Transcriptional analysis revealed that the *sar* locus is composed of three overlapping transcripts designated *sarA*, *sarC* and *sarB* (sizing at 0.58, 0.84 and 1.15kb respectively) (Bayer *et. al.,* 1996). These three transcripts terminate in a common 3' end but originate from three distinct promoters. Complementation analysis indicated that one of the genes (*sarA*, the 0.58-kb transcript) within the *sar* locus appears to be necessary for the optimal expression of RNAIII in the *agr* locus (Heinrichs *et. al.,* 1996).

sar is implicated in controlling expression at the transition, switching off transcription of target genes and activating transcription of *agr*. SarA is actually a transcription factor for *agr* P2 and P3 (Morfeldt *et. al.,* 1996) but also regulates exoprotein genes independently. In fact, SarA functions as a major repressor of protease activity. Several proteases, including a V8 serine protease and a novel metalloprotease, were identified by N-terminal sequencing as the major extracellular proteins repressed by SarA (Chan and Foster, 1998). In kinetic studies, a mutation in

sarA delayed the onset of α-haemolysin expression and reduced the levels of *hla* to approximately 40% of the level in the parent strain. It has been determined that *sar* is the primary regulatory element controlling the expression of *cna* in *S. aureus*, and the regulatory effects of *sar* and *agr* on *cna* transcription are independent of the interaction between *sar* and *agr* (Gillaspy *et. al.,* 1998).

1.5.3 In vivo significance of *sar* and *agr*

Strains deficient in *agr* were shown to have reduced fibrinogen-binding activity (Wolz *et. al.,* 1996). Both *agr* and *sar* mutants have been tested in animal models of virulence. In almost all models reduced virulence was observed. These include a mouse model of septic arthritis (Abdelnour *et. al.,* 1993, Nilsson *et. al.,* 1996), a rabbit model of osteomyelitis (Gillaspy *et. al.,* 1995), and a rabbit model of endocarditis (Cheung *et. al.,* 1994b). The fact that an *agr/sar* double mutant is more impaired in virulence factor production than either *agr* or *sar* single mutants implies that *sar* also has a role in the expression of virulence factors independent of *agr*.

1.6 Aims of project

The aims of this project were to make a panel of monoclonal antibodies (mAbs) against the Clf33(221-550) protein, and to identify mAbs which interfered with the ClfA-fibrinogen interaction. This was performed to help further characterise the ClfA protein at the molecular level and possibly to aid in localization of the ligand-binding sites of ClfA. A panel of mAbs were also made against the Clf42(332-559) protein. This protein has the ability to neutralize the blocking effect of anti-Clf33(221-550) polyclonal antiserum (McDevitt *et. al.,* 1995b). Polyclonal antiserum raised against Clf42(332-559) also has the ability to block clumping (McDevitt, Personal communication). Inhibitory mAbs identified were subsequently to be used in passive immunization in studies in an animal model of *S. aureus* infection in order to determine if anti-ClfA antibodies are protective in vivo.

CHAPTER 2

MATERIALS AND METHODS

2.1 *S. aureus* strains

S. aureus strain Newman with a deletion in the gene for protein A (Newman Δ*spa*) was used for all bacterial clumping and adherence experiments and also for the platelet-fibrin clot adherence experiment. A clinical isolate known as strain F. Barnett was used for the mouse renal infection model. These strains and the four other strains used for the cell blot assay are listed and described in Table 2.1.

2.2 Recombinant protein expression

Recombinant ClfA proteins (Table 2.2) were expressed in *E. coli* XL-1 blue using the pKK233-2 (Amman and Brosius, 1985) or pQE30 expression system (Quiagen) as specified. Starter cultures of *E. coli* XL-1 blue containing the plasmid of interest were grown in Luria broth overnight at 37°C and 200 r.p.m. This was diluted 1:100 and grown for 4 hours as for the starter culture until A_{600} was between 0.5-0.75. The cells were then induced using 0.5mM IPTG and grown for a further 4 hours. Cells were harvested at 10,000xg and washed in 50ml PBS and resuspended in 1/50 of bulk culture volume with binding buffer which included complete protease inhibitor cocktail tablets (Boehringer Mannheim) used according to the manufacturer's instructions.

2.3 Nickel-chelate affinity chromatography

Protein was purified from cell lysates according to the manufacturer's instructions using nickel-chelate affinity chromatography. Cells were lysed either by passage through a French pressure cell at 2000 p.s.i. Alternatively, sonication on ice was performed using 5x7μ cycles of 30s each, with 2 min cooling in between. The lysate was then centrifuged at 18,000xg and 4°C for 1h to pellet whole cells and cell debris. The chromatography step was carried out using a C-10/10 column (Pharmacia) and Sepharose 6B resin coupled to iminodiacetic acid (Sigma). 2ml of resin was used for every 1l of original culture induced. The resin was charged with 700mM NiCl (Sigma) and excess solution was washed off with three bed volumes of binding buffer (5mM Imidazole, 0.5M NaCl, 20mM Tris-HCl pH7.9). A further three bed volumes of binding buffer were then run through to ensure the column was equilibrated. The cleared cell lysate was added using a P-1 peristaltic pump (Pharmacia). The column was then washed with binding buffer until the A_{280} of the eluate was 0.01. The protein was then eluted using elute buffer (1M Imidazole, 0.5M NaCl, 20mM Tris-HCl pH7.9).

Table 2.1. *S. aureus* strains used in this study.

S. aureus strain	description	source/reference
Newman	High level of clumping factor	Duthie and Lorenz, 1952
NewmanΔ*spa*	Protein A mutant of Newman	McDevitt et. al., 1995
Newman *clfA*$^-$	Clumping factor mutant of Newman	McDevitt et. al., 1994
NewmanΔspa *clfA*$^-$	Clumping factor and protein A mutant	McDevitt et. al., 1995
8325-4	Very low level of clumping factor	Novick, 1967
F. Barnett	ClfA positive clinical isolate	J. Patti, Houston, Texas.

Table 2.2. Recombinant ClfA proteins used in mAb binding studies. Fibrinogen-binding truncates are in bold type. All constructs were made in pQE30 except for the underlined truncates, which were made in pKK233-2 (McDevitt *et. al.,* 1995).

Recombinant truncate	residues
Clf27	420-550
Clf28	23-424
Clf29	23-308
Clf33	221-550
Clf40	40-559
Clf41	221-559
Clf42.	332-559
Clf43	221-484
Clf44	272-559

2ml elute fractions were collected by hand and analysed by sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE) (Laemmli, 1970), and Western immunoblotting using anti-Clf33(221-550) polyclonal antiserum (1:1000) as previously described (McDevitt *et. al.*, 1994).

2.4 Gel filtration chromatography

Gel filtration chromatography was used to further purify Clf33(221-550) and Clf42(332-559) proteins for use in rabbit and mouse immunisations and also ELISA (Porath and Flodin, 1959). Positive fractions from nickel-chelate affinity chromatography were pooled and concentrated to 1-4ml using a Fugisep maxi protein concentrator (Intersep). A Sephacryl HR 200S C-16/100 column (Pharmacia) was loaded with the concentrated protein sample and run at 2ml/h at 4°C. 4ml fractions were collected using a Redifrac 200 fraction collector (Pharmacia) and analysed using SDS-PAGE and Western immunoblotting (see section 2.3).

2.5 Polyclonal antibody production

New Zealand white rabbits were given an initial immunisation of 25μg of purified recombinant Clf33(221-550) subcutaneously in Freund's complete adjuvant. Two subsequent boosters were given with 25μg of protein in Freund's incomplete adjuvant. The rabbits were bled out two weeks after the final booster. Antibody titers were checked by Western immunoblot, and the IgG fraction isolated from the serum by ammonium sulphate precipitation. Following precipitation, the IgG fraction was prepared and stored as previously described (Owen, 1995).

2.6 Protein G-sepharose affinity chromatography

Anti-Clf33(221-550) polyclonal serum and mAbs from the Clf33(221-550) fusion were affinity purified to obtain the IgG fraction using Protein G-sepharose (Harlow and Lane, 1988). 5ml of protein G-sepharose (Sigma) was packed into a C-10/10 column and washed three times with PBS to equilibrate the column. The IgG-containing sample containing was then applied and excess protein washed off with PBS. Washing was continued until the A_{280} of the eluate returned to baseline value. The IgG was then eluted using 0.1M Glycine pH 3.0. 2ml fractions were collected by hand into tubes containing 200μl 1.0M Tris-HCl pH 9.0 in order to neutralize the elute buffer and

prevent antibody denaturation. Fractions were analysed by indirect ELISA (see section 2.9). Positive fractions were pooled and dialysed against PBS at 4°C. Purified IgG was stored at 4°C in the presence of 15mM NaN_3 or in aliquots at -20°C.

2.7 Protein concentration determination

The standard Bradford microassay was used (Bradford, 1976). A standard curve was constructed using fibrinogen at concentrations between 1-25μg. Test proteins were then diluted appropriately and protein concentration was estimated using the standard curve.

2.8 Monoclonal antibody production

2.8.1 Mouse immunisation

Six week old female balb/c mice were immunised by a standard protocol (Harlow and Lane, 1988). Mice received an initial subcutaneous immunisation with 25μg/ml of purified protein in Freund's complete adjuvant. Three subsequent immunisations with 25μg/ml protein were given at 12-14 day intervals in Freund's incomplete adjuvant. 3-4 days prior to fusion, the mice were given 25μg of protein in PBS without adjuvant via the intravenous route.

2.8.2 Preparation of myeloma cells

Prior to fusion, growing Sp2/0 myeloma cells (Immune Systems) were diluted 1:10 and subcultured into 4 fresh 75cm^2 flasks (Grenier). The cells were fed with growth medium after 3 days, and used for fusion on the 6th day. On the day of fusion, flasks were shaken by hand to remove the cells and cells were then harvested from the medium by centrifugation at 1000 r.p.m. for 5 min. Cells were washed in 10-15ml of wash medium (RPMI 1640 with L-Glutamine and without HEPES, Gibco), spun at 1000 rpm for 5 min and resuspended in 5ml wash medium. Cells were then counted using a Neubauer chamber and kept at 37°C until needed.

2.8.3 Preparation of splenocytes

5ml of wash medium was pipetted into a sterile petri dish. The spleen was disected from the mouse under aseptic conditions and placed in the dish. Blood was also taken from the mouse in order to check the immune response by indirect ELISA.

The spleen was then disrupted using a forceps in order to make a crude cell suspension. This suspension was then pipetted against a sterile strainer into a 50ml tube (Grenier). Any lumps of tissue remaining were washed through and disrupted using additional wash medium until a final volume of 20ml was reached. The suspension was left to stand for 3 minutes to pellet large clumps. The supernatant was carefully removed into a fresh 50ml tube and centrifuged at 1000 x r.p.m. for 5 min. The splenocytes were resuspended in 5ml of wash medium and counted as for the myeloma cells.

2.8.4 Fusion procedure

Splenocytes from immune mice were mixed with Sp2/0 myeloma cells at a ratio of 10:1 according to the manufacturer's protocol, using a minimum of 10^7 Sp2/0 cells. Cells were spun at 1,200 r.p.m. for 5 min, resuspended in 5ml wash medium and the wash step repeated. The supernatant was then removed taking care not to disrupt the cells while removing as much fluid as possible. 1ml PEG 4,000 (Gibco) prewarmed at 37°C was then added to the cell mixture. The cells and PEG were swirled gently together and 0.5ml HAT/feeder medium was added. 3 subsequent 1ml amounts of medium were added at 1 min intervals and the mixture swirled. Finally, 5ml of medium was added, the cap of the tube replaced and the suspension mixed gently but thoroughly. It was then spun at 500 r.p.m. for 5 min. The supernatant was removed and the fused cells were resuspended in 10ml HAT/feeder medium.and incubated for 15 min at room temperature. The cells were then diluted 1/10 in 90ml HAT/feeder medium and 10x96 well plates containing 100µl prewarmed medium were seeded. The plates were incubated in a humid 5%CO_2 atmosphere for 12 days undisturbed. Supernatant was then assayed for mAbs reacting with Clf33(221-550) or Clf42(332-559) by indirect ELISA (see section 2.9). Positive clones were expanded into 24-well plates, 25cm^2 flasks and finally 75cm^2 flasks.

For mAbs raised against Clf33(221-550), the growth medium used was RPMI 1640 containing Glutamax I (Gibco) supplemented with 20% foetal bovine serum (Medical Supply Company), 50x HAT supplement (Gibco), 100U/ml penicillin, 100µg/ml streptomycin and 25µg/ml amphotericin B (Sigma) and 5% Briclone feeder supplement (BioResearch Ireland). A serum-free medium was used for the fusion with the Clf42(332-559) truncate. The 20% foetal bovine serum was replaced with 1% nutridoma SR or nutridoma SP (Boehringer Mannheim).

2.9 Indirect ELISA for mAb screening

Specific antibody-secreting clones were identified by indirect ELISA (enzyme-linked immunosorbent assay) screening. A modification of a method described in (Newell *et. al.,* 1988) was used. 96-well plates were coated overnight with purified ClfA protein diluted to between 3-10μg/ml in carbonate buffer (15mM Na_2HCO_3, 35mM $NaH(CO_3)_2$, 3.2mM NaN_3,pH9.6). Plates were incubated in blocking solution, 2% bovine serum albumin (BSA) in phoshate buffered saline (PBS, pH 7.3) containing 0.05% (vol/vol) Tween 20) for 60min at 37°C. Plates were washed three times with wash buffer (0.145M NaCl, 0.05% (vol/vol) Tween 20) in a Multiwash Plus ELISA plate washer (Labsystems). Cell culture supernatants were then added and incubated at 37°C for 2h. Plates were washed again as above and incubated with goat anti-mouse immunoglobulins coupled to horseradish peroxidase (Dakoplatts, 1:2000) for 30 min. After being washed, 100μl per well of chromogenic substrate (580μg/ml tetramethylbenzidine, 0.0001% H_2O_2 in 0.1M sodium acetate buffer pH5.0) was added and allowed to develop for 10 min. The reaction was stopped by the addition of 50μl of 2M H_2SO_4. Plates were read at 450nm in an ELISA plate reader (Labsystems Multiskan Plus).

2.10 Isotype analysis

Isotyping of monoclonal antibodies was performed using a mouse monoclonal antibody isotyping kit (Serotech) based on haemagglutination of red blood cells coupled to antibodies specific for each subclass of mouse immunoglobulin (Coombs, 1987). The kit was used according to the manufacturer's instructions. Tissue culture supernatant was diluted 1/50 in an eppendorf tube to give a final concentration of approximately 1μg/ml. 30ml of this dilution was placed in each of 8 wells across a 96-well U bottomed microtitre plate. 30μl of each isotype-specific reagent was added to the wells. The plate was covered and tapped gently to mix, and left at room temperature on a flat surface for one hour for results to develop. A full or partial carpet of cells was taken as a positive result, where a small red circle or button was negative, as shown by positive and negative controls provided with the kit.

2.11 Whole cell dot-blot assay

S. aureus mutant strains used are listed in Table 1.3. Bacterial overnight cultures were grown in 2ml Tryptic Soy Broth at 37°C with shaking at 200 r.p.m. The cells were then washed once with PBS, concentrated twofold and 5ml of each suspension was spotted onto a polyvinylidinedifluoride (PVDF) membrane (Boehringer Mannheim). After drying, the membrane was blocked in 2% skim milk solution for 2h. Anti-Clf33(221-550) polyclonal serum at a 1:1000 dilution and preimmune serum from the same rabbit at 1:1000 and 1:100 dilutions were then incubated with the immobilised cells. Membranes were washed as for the Western immunoblotting protocol. Bound antibody was detected using Protein A conjugated to horseradish peroxidase (Sigma) at a 1:1000 dilution. Blots were developed as for Western immunoblots.

2.12 Monoclonal antibody purification

Clf33(221-550) mAbs were initially purified from bulk-grown 50ml tissue culture supernatants in $75cm^2$ flasks by polyvinylpyrollidone clarification and subsequent protein G-sepharose (Sigma) affinity chromatography (Harlow and Lane, 1988). It was necessary to remove the bovine fibrinogen from the Clf33(221-550) mAb preparation in order to assay for blocking activity. As the Clf42(332-559) fusion was performed using fibrinogen-free cell culture medium, mAbs reactive against Clf42(332-559) on ELISA did not need to be affinity-purified for initial screening to identify blocking antibodies. 10ml Clf42(332-559) mAb supernatants were subject to ammonium sulphate precipitation for 4h at 4°C. Supernatants were then spun and the precipitate resuspended in 1ml of PBS. This was then dialysed against three changes of PBS. This preparation was then assayed for blocking activity. For large-scale production of mAbs 6, 36 and 47 and a negative control IgG1 (mAb35, a non-blocking mAb raised against Clf33(221-550)), mAbs were purified using gel filtration chromatography on Sephacryl HR 200S. 100ml of tissue culture supernatant was spun at 3000xg for 30 min to remove cell debris. The supernatant was precipitated using ammonium sulphate precipitation at 4°C overnight. The supernatant was spun at 3000xg for 30 min at 4°C and resuspended in 10ml PBS. Yield was increased by omitting the precipitation step. Alternatively, 100ml tissue culture supernatant was passed through a 0.2mM filter and subsequently concentrated 10-20-fold to 5-10ml using a fugisep maxi protein concentrator (Intersep). This was loaded onto the gel

filtration column. 4ml fractions were collected using a Redifrac 200 fraction collector (Pharmacia), and 50µl of each fraction was analysed by indirect ELISA. Antibody-positive fractions were pooled and concentrated using a fugisep concentrator. Antibody activity was then checked again using the indirect ELISA, and protein concentration was determined.

2.13 Soluble clumping assay

This assay was performed as previously described (McDevitt *et. al.,* 1994). Briefly, serial twofold dilution of 1mg/ml human fibrinogen (Kabi, Stockholm) were made in PBS in a microtiter tray. $2x10^7$ c.f.u. *S. aureus* strain Newman Δ*spa* cells were added to each well. The highest dilution of fibrinogen where clumping occured after 5 minutes was the minimum clumping dose (CD). For clumping inhibition, serial twofold dilutions of mAbs or polyclonal IgG were made. Each dilution was incubated with $2x10^7$ c.f.u. of bacterial cells for 10 min. Fibrinogen was then added (2x CD) and the lowest dilution where inhibition occurred was defined as the minimum inhibitory dose (ID).

2.14 Bacterial adherence assay to solid-phase fibrinogen

The following modifications of a method used by Wolz and co-workers (Wolz *et. al.,* 1996) were made. Polystyrene microtiter plates were coated overnight at 4°C with 5µg/ml of human fibrinogen (Calbiochem). Plates were washed between incubations as for direct ELISA. Plates were blocked at 37°C for 1h in 2% BSA in PBS with 0.05% (vol/vol) Tween 20. Simultaneously, $5x10^8$ of washed *S. aureus* Newman Δ*spa* cells were incubated with serial doubling dilutions of polyclonal antiserum (starting 1:100) or purified monoclonal IgG starting at a final concentration of 1mg/ml. 100µl of these mixtures were then added to the wells, and incubated for 2h. After gentle washing by hand, adherent cells were fixed by adding 100µl/well of 25% formaldehyde for 30 min. Cells were then stained with crystal violet for 1 min, washed, and the plate was read in an ELISA plate reader at 570nm.

2.15 Inhibition of fibrinogen binding to solid-phase Clf41(221-559)

ELISA plates were coated overnight with 10µg/ml of Clf41(221-559) in carbonate buffer. Plates were blocked as for the indirect ELISA. After washing, serial

twofolds of mAbs starting 500μg/ml were added to the plated and incubated at 37°C for 2h. Plates were washed and 100μl per well of 5μg/ml of purified human fibrinogen was added for 1h at 37°C. The plates were again washed and the amount of fibrinogen bound to Clf41(221-559) was detected using anti-fibrinogen polyclonal serum coupled to horseradish peroxidase (Dakoplatts 1:1000). A chromogenic substrate was then added as for the indirect screening ELISA.

For the mAb specificity ELISA, wells were coated with 10μg/ml recombinant protein, and all steps were as for the indirect screening ELISA. Data was expressed as % binding to Clf41(221-559) for mAbs 6, 36 and 47, and for anti-Clf33(221-550) polyclonal IgG. Binding of anti-ClfB(45-542) and anti-SdrC polyclonal serum was expressed as a % binding to recombinant ClfB(45-542) and SdrC respectively.

2.16 Plasma clot adherence assay

A modification of a previously described assay was used (Moreillon *et. al.*, 1995, Rampyari *et. al.*, 1990). Canine blood containing 10% sodium citrate buffer (Sigma) was centrifuged at 3000xg for 10 min at room temperature. The cleared plasma was then transferred to a clean tube. Platelet-fibrin clots were made by aliquoting 0.5ml volumes of plasma into 35mm tissue culture plates followed by 100ml of 0.2mM $CaCl_2$ and 100ml of bovine thrombin (500 NIH-U/ml, Sigma) . The plates were mixed by hand and allowed to coagulate at room temperature. Bacterial adherence was determined as follows. 2ml of PBS containing $5x10^3$ c.f.u./ml was added to the clots and shaken for 3 min on an orbital shaker. The inoculum was decanted and the clots washed twice for 5 min each with 2ml PBS. The clots were then overlayed with 3ml Tryptic Soy agar, allowed to set and incubated for 15h at 37°C. Counts obtained were expressed as a percentage of the input inoculum. Inhibition of bacterial attachment to the clots was performed as follows. Bacteria were preincubated with anti-Clf33 polyclonal antiserum (1:100) or mAbs 36 and 47 (20μg/ml total antibody concentration) before addition to the clots. All other steps are as for the basic adherence assay.

2.17 Mouse renal infection and bacteraemia model

Initially, two pilot studies were performed. The first was done in order to determine the minimum dose required in order to establish renal infection of the mouse.

Three mice were injected by the intraperitoneal route with *S. aureus* strain F. Barnett at $6.0x10^6$, $6.0x10^7$ and $6.0x10^8$ c.f.u. in 0.5ml PBS. After 48h, these mice were sacrificed, the kidneys homogenised and plate counts performed. When it was determined that only the $6x10^8$ dose caused infection, three more animals were given $1.4x10^9$ c.f.u. in 0.5ml PBS each in order to establish that infection was reproducible.

The second pilot study was performed in order to determine that mAbs 36 and 47 persisted in the bloodstream of the mice over the duration of the experiment. One mouse received an intramuscular injection of 250µg mAb36 + 250µg of mAb47. Blood samples were taken from the tail vein at 24 and 48h timepoints, and the mice were sacrificed at 120h after injection with the mAbs. Blood samples were analysed by indirect ELISA.

For the actual infection experiment, five mice were immunised with 1mg mAbs36+47 (500µg of each mAb) intramuscularly and five mice with 1mg of control IgG1. Forty-eight hours later, all ten mice were then challenged with $4x10^8$ c.f.u. in 0.5ml PBS by the intraperitoneal route. Mice were then sacrificed after 48h, kidneys homogenized, diluted and plated and qualitative blood cultures were also performed.

2.18 Western immunoblotting of recombinant truncated ClfA molecules

This was performed as described by (McDevitt *et. al.,* 1994) with the following exceptions for blots probed with mAb cell culture supernatants. SDS-PAGE gels containing *E. coli* induced lysates or purified protein was transferred to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad) in the presence of semi-dry transfer buffer (25mM Tris.HCl, 192mM Glycine, 20% Methanol, pH 8.3) for 1h at a current limit of 0.57A and a voltage of 20V. When transfer was completed, blots were blocked in 10% skim milk in PBS for 2h at room temperature with shaking or alternatively overnight at 4°C. Membranes were then incubated with ELISA-positive cell culture supernatants for 2h with shaking at room temperature and washed with three changes of PBS. Goat anti-mouse immunoglobulins coupled to horseradish peroxidase diluted in blocking solution were added (Dakoplatts, 1:1000). The blot was washed and then developed in zymogram solution (50ml PBS, 25mg of 4-chloro-1-napthol in 15ml methanol and 40µl H_2O_2).

2.19 Binding of polyclonal IgG and mAbs to solid-phase peptides

The peptides used were 25-residues overlapping by 5 corresponding to 333-557 and also a 11-residue peptide corresponding to residues 310-321 of ClfA (Table 2.3). These were provided by the Texas A&M University, Houston, Texas. ELISA plate wells were coated overnight at room temperature with 100μg/ml of peptide. Plates were washed between incubation steps as for the indirect ELISA. Wells were blocked with 0.1% BSA with 0.05% Tween-20 in PBS for 2h at room temperature. Anti-Clf33(221-550) polyclonal IgG 1:1000, mouse serum 1:500 or mAb supernatant positive on an indirect ELISA was added and incubated for 2h at 37°C. Bound antibody was detected using goat anti-rabbit HRP 1:1000 or rabbit anti-mouse HRP 1:2000 respectively. Chromogenic substrate used and plate reading were as for the indirect ELISA.

2.20 ELISA for binding of pAb and mAb to site-directed mutants of ClfA

Mutant proteins used in this assay are described in Table 2.4. The concentration of each protein was standardised using the Bradford protein microassay. ELISA wells were coated with 10μg/ml of protein in carbonate buffer pH 9.6 overnight at room temperature. Plates were blocked and washed as for the indirect ELISA. Tissue culture supernatant containing mAbs or anti-Clf33(221-550) polyclonal IgG 1:1000 was then added to the wells and incubated for 2h at 37°C. Bound antibody was detection and substrate development was performed as for the indirect ELISA.

2.21 Inhibition of mAbs 36 and 47 binding to Clf41(221-559) by an EF hand (310-321)peptide

All steps were performed as for the indirect ELISA with the exception that 10μg/ml mAbs 36 and 47 were preincubated with 100μg/ml of a peptide corresponding to ClfA residues 310-321 for 1h before addition to Clf41(221-559) coated ELISA wells. Data was expressed as % of mAbs 36 and 47 binding in the absence of a peptide.

2.22 Binding of mAbs to Clf41(221-559)-fibrinogen and Clf41(221-559)-fibrinogen γ chain peptide complexes

A 10μg/ml solution of Clf41(221-559) was prepared. This was diluted 1:2 in an eppendorf tube with equimolar amounts of whole fibrinogen (Calbiochem), a peptide

Table 2.3. Soluble overlapping synthetic peptides corresponding to ClfA residues 333-557 used in mapping studies.

peptide	sequence	residues
clf-1	LTMPA YIDPE NVKKT GNVTL ATGIG	333-357
clf-2	ATGIG STTAN KTVLV DYEKY GKFYN	353-377
clf-3	GKFYN LSIKG TIDQI DKTNN TYRQT	373-397
clf-4	TYRQT IYVNP SGDNV IAPVL TGNLK	393-417
clf-5	TGNLK PNTDS NALID QQNTS IKVYK	413-437
clf-6	IKVYK VDNAA DLSES YFVNP ENFED	433-457
clf-7	ENFED VTNSV NITFP NPNQY KVEFN	453-477
clf-8	KVEFN TPDDQ ITTPY IVVVN GHIDP	473-497
clf-9	GHIDP NSKGD LALRS TLYGY NSNII	493-517
clf-10	NSNII WRSMS WDNEV AFNNG SGSGD	513-537
clf-11	SGSGD GIDKP VVPEQ PDEPG EIEPI	533-557
EF peptide	DSDGNVIYTFTD	310-321

Table 2.4. List of deletion and site directed mutants used in mAb binding studies.

construct	mutation	reference
Clf51	40-309,322-559 (Δ310-321)	O'Connell et. al., 1998
Clf68	D310A/D312A/T318A/D321A	O'Connell et. al., 1998
Clf92	D443A/S445A	O'Connell et. al., 1998
SM4	E526L/V527S/A528R/G532Z	Hartford, Personal communication

corresponding to residues 396-411 of the γ-chain of fibrinogen or PBS as a negative control. These mixtures were kept for 1h at room temperature. ELISA wells were then coated with Clf41(221-559) in PBS or the Clf41(221-559) complexes. Blocking and washing were done as previously described. A 1:1000 dilution of pAb or ELISA reactive tissue culture supernatants were added. These were incubated for 1h at 37°C. Bound antibody was detected as previously described. Binding of an antibody to uncomplexed Clf41(221-559) was considered to represent 100% binding activity. Binding to the Clf41(221-559)-γ chain peptide and Clf41(221-559)-fibrinogen complexes was calculated as a percentage of this.

2.23 Sequence analysis of ClfA residues 221-559

Antigenicity index of ClfA 221-559 was predicted according to the method of Jameson and Wolf (Jameson and Wolf, 1988). This was done using plot structure in the Wisconsin package of programmes, version 8 (Genetics Computer Group, Madison, WI, USA).

2.24 Statistical analysis

A one tailed t-test was performed in order to determine statistical significance. This was done using a Biostatistics software package for the Apple Macintosh.

CHAPTER 3

PRODUCTION AND IDENTIFICATION OF ANTI-CLUMPING FACTOR A MONOCLONAL ANTIBODIES WHICH BLOCK THE CLUMPING FACTOR A-FIBRINOGEN INTERACTION

3.1 Introduction

In August 1975 George Kohler and Cesar Milstein published a paper in Nature describing the continuous culture of fused cells secreting antibody of predefined specificity (Kohler and Milstein, 1975). The last sentence of this paper stated that such cultures could be valuable for medical and industrial use. The discovery of this technique resulted in Nobel prizes for the authors in 1984. Since then, developments in monoclonal antibody production and their application have had profound implications on biology in general.

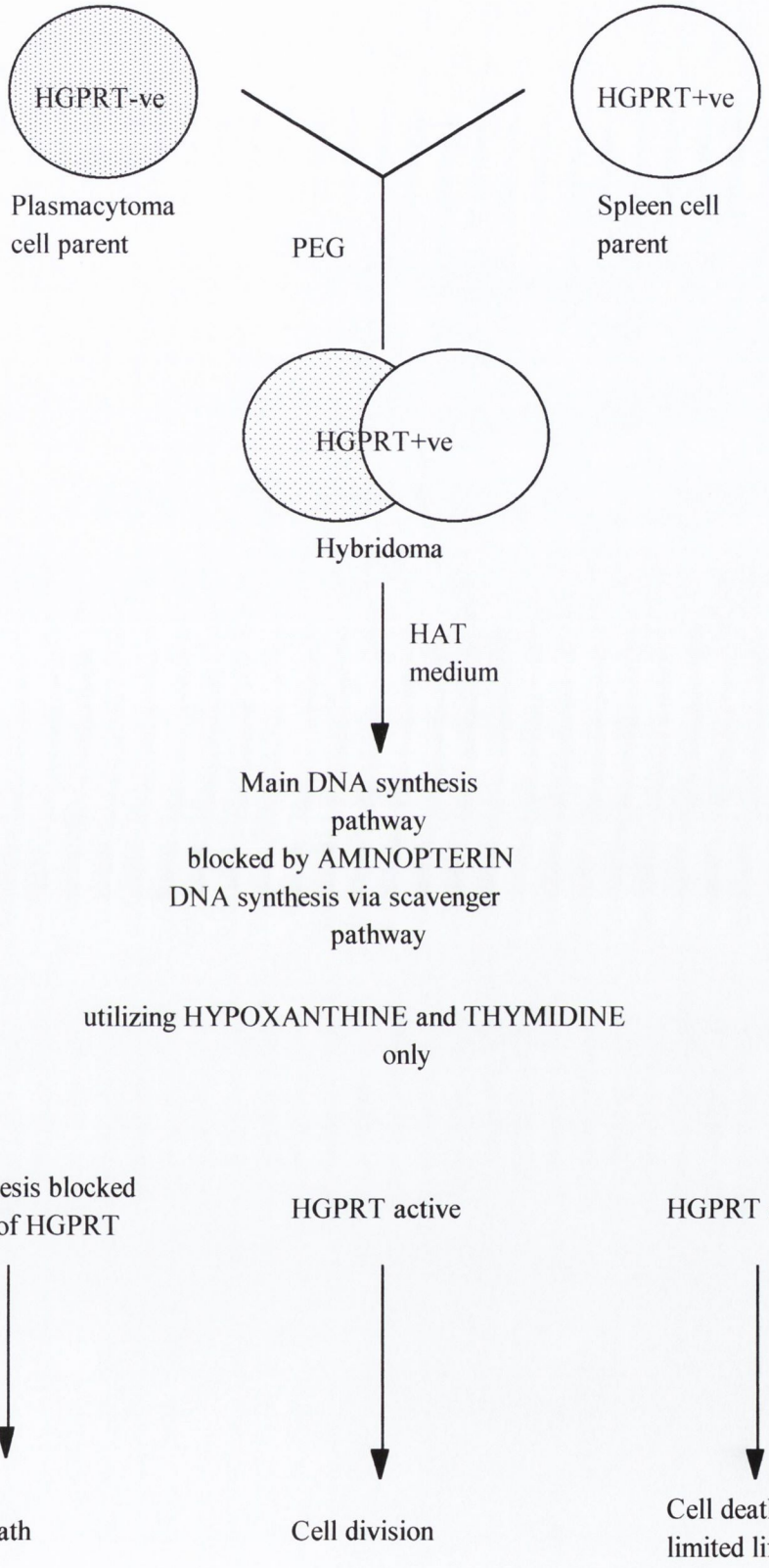
Monoclonal antibodies (mAbs) can now be obtained in virtually unlimited quantities and specific for any antigen by fusing myeloma cells with lymphocytes raised against that antigen. A clone of the resulting hybridoma cell synthesizes the lymphocyte's immunoglobulin but has the myeloma cell's immortality. Monoclonal antibodies have become indispensable biomedical tools. They can be used to detect extremely small amounts of almost any specific biological substance. For example, they have made possible the routine testing of blood for the presence of HIV thereby protecting the public blood supply.

3.1.1 Monoclonal antibody production.

The procedure of monoclonal antibody production involves the fusion of an immune spleen cell with a myeloma cell in the presence of polyethylene glycol (Fig. 3.1). The resulting hybrid is grown in a selective medium containing hypoxanthine, aminopterin and thymidine (HAT medium). The aminopterin component of the medium blocks the main DNA synthesis pathway. Therefore, the scavenger pathway using only hypoxanthine and thymidine must compensate. This precludes the growth of non-hybrid myeloma cells as their scavenger pathway is not functional. Unfused B-lymphocytes gradually die as they are not immortal, so all that remain are fused cells.

Hybridoma clones secreting antibody of the required specificity must then be identified by screening of the cell culture medium from these clones for mAbs reactive against the antigen of interest. This can be done by several methods, including ELISA and immunoblotting. Once these important clones are identified, they can be subcloned into larger culture vessels. This makes enough cells available for preservation in liquid nitrogen, a procedure that is important to carry out at the earliest possible time. The clone can then be grown in bulk in order to generate large amounts of antibody. Alternatively, the clone can be injected into the peritoneal cavity of a mouse in order to

Figure 3.1. Selection of hybridoma cells.


HGPRT-ve
Plasmacytoma cell parent
PEG
HGPRT+ve
Spleen cell parent
HGPRT+ve
Hybridoma
HAT medium
Main DNA synthesis pathway blocked by AMINOPTERIN
DNA synthesis via scavenger pathway
utilizing HYPOXANTHINE and THYMIDINE only
DNA synthesis blocked in absence of HGPRT
HGPRT active
HGPRT active
Cell death
Cell division
Cell death due to limited lifespan

produce ascities fluid rich in specific antibody. However, this practice is becoming increasingly rare due to changes in animal welfare laws (Marx *et. al.,* 1997).

3.1.2 Applications of monoclonal antibodies.

There are many applications of monoclonal antibodies in research and also for diagnostic and therapeutic use. They can be used to show if antigens share cross-reactive epitopes, and thus define an evolutionary relationship between reactive molecules as defined by a specific mAb. For example, in a search for common pneumococcal proteins, mAbs were produced against clinical isolates of *Streptococcus pneumoniae* (Kolberg and Sletten, 1996). Two mAbs were isolated which bound to a 40kDa protein from extracts of *S. pneumoniae* cells. These mAbs were used to isolate the protein that showed 90% homology with a surface-located glyceraldehyde-3-phosphate dehydrogenase from *S. pyogenes*. Conserved epitopes can also occur between distantly related species. The laminin binding epitope on gp43 from eukaryotic *Paracoccidioides brasiliensis* is recognized by a mAb raised against *S.aureus* laminin receptor (Vincentini *et. al.,* 1997). This finding reinforces the possibility of evolutionary conservation of such epitopes. The relationship between the integrin $\alpha_M\beta_2$ I(A) domain insertion in the α-subunit and the fibrinogen-binding integrin-like protein on the surface of *Candida albicans* has been shown using a mAb (Gale *et. al.,* 1996). mAbs raised against this integrin cross-reacted with the αInt1p protein of *C. albicans*. This is relevant to ClfA, as ClfA is a fibrinogen-binding protein with some homology to αInt1p.

Species-specific mAbs are also extremely useful in the diagnosis of bacterial infection. For example, *Pasteurella multocida* is characterized serologically by identification of capsular antigens (Vasfi *et. al.,* 1995). This organism is the causative agent of atrophic rhinitis in pigs worldwide, the serotype D strain being the most prevalent. mAbs have been produced which specifically identify pathogenic strain varieties of many different organisms, and could potentially be used to generate important data in epidemiological studies. Also, enterotoxigenic *Escherichia coli*, a major cause of diarrhoea in developing countries, posesses a colonisation factor known as coli surface antigen 6 (CS6) on its cell surface (Helander *et. al.,* 1997). CS6 is the most frequently identified surface antigen on clinical isolates. A mAb that identified this antigen has been produced and shown to be extremely important in the diagnosis of *E. coli* strains containing this protein. Also, this mAb did not recognise any CS6 non-

producing strains. This test could not be done previously using polyclonal serum due to non-specificity. In fact, some mAbs are so specific that they can differentiate the repetitive elements which are highly homologous but not identical in structure within the same adhesin, as seen with mAbs generated against the p50 adhesin of *Mycoplasma hominis* (Henrich *et. al.,* 1996).

3.1.3 Therapeutic uses of antibodies.

Antibody-based therapy was used in the early 20th century to treat a variety of human infections including those caused by *Corynebacterium diphtheriae*, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Clostridium tetani* (Casadevall, 1996). This method of treatment was generally abandoned when antimicrobial chemotherapy became widely practised. However, antibody therapy is still used today in several clinical situations, such as for post-exposure prophylaxis against several viral diseases (rabies, measles, hepatitis A and B) and toxin neutralization (diptheria, tetanus and botulism). Although antibodies are generally more useful in the prevention of infection rather than in the treatment of a disease that is already established in a host, therapy using mAbs may have important applications in the future. This is especially important when the threat of antibiotic resistant bacterial strains is considered.

In some cases, due to the specific nature of their binding, mAbs may also be more useful than polyclonal antibody preparations which have been used previously in the treatment of several infections (Casadevall, 1996). For example, 0.7mg of two human mAbs recognising tetanus toxin have the same activity as 100-170mg of immune globulin (Lang *et. al.,* 1993). Potentially therapeutic mAbs can mediate antimicrobial function through a variety of mechanisms, including inhibition of microbial attachment, agglutination, viral neutralization, toxin neutralization, antibody-directed cellular cytotoxicity, complement activation and opsonization (Heinzel, 1995). Some IgA mAbs can neutralize intracellular viruses (Manzanec *et. al.,* 1992) and a mAbs to *Toxoplasma gondii* have been reported to interfere with intracellular replication of the parasite (Mineo *et. al.,* 1994). Attempts have also been made to treat Gram negative bacterial septic shock by employing mAbs that bind to cytokines (Bobmer *et. al.,* 1993).

The realisation that antibody fragments can be expressed on the surface of bacteria (Stahl and Uhlen, 1997) or bacteriophage particles (Hoogenboom, 1997) has revolutionized the ability to mimic B-cell immunity in vitro. Very large collections of

antibody molecules or their variable domains can be expressed on the surface of filamentous bacteriophages so that antibodies with desired specificities and high affinities can be obtained by screening a library against the antigen of choice. The ease with which mAbs or antibody variable fragments can be generated demonstrated that they may play an even more important role as tools or therapeutic agents in biology in the future. However, disadvantages also exist in using antibodies for therapy, such as the formation of anti-idiotype antibodies or rapid clearance before an antibody can act against its target.

3.1.4 Objective of this study

This work describes the production of mAbs against the minimum ClfA fibrinogen-binding truncate Clf33(221-550) and the non-fibrinogen binding Clf42(332-559). Although the Clf42(332-559) truncate does not bind fibrinogen, it has the capability of neutralising the inhibitory effect of anti-Clf33(221-550) polyclonal serum (McDevitt *et. al.,* 1995). This would suggest that this protein contains immunologically important epitopes. Also, it was not possible that mAbs would be generated to a ClfA-fibrinogen complex on immunisation with Clf42(332-559). This was a concern when using Clf33(221-550) as an immunogen, as *S. aureus* Newman is capable of forming clumps in mouse fibrinogen. The mAbs generated against these recombinant ClfA proteins were screened for their ability to block the ClfA-fibrinogen interaction. A solid-phase *S. aureus* adherence assay was also adapted and modified from a previous study. This assay, in combination with a soluble bacterial clumping assay, was used in order to identify mAbs with inhibitory activity.

3.2 Results

3.2.1 Screening for mAbs which inhibit bacterial adherence to solid-phase fibrinogen

An ELISA plate adherence assay was adapted from a previous study (Wolz *et. al.,* 1996). Initially, a standard curve (A_{570} vs bacterial cell number) was constructed in order to determine the minimum amount of bacterial cells expressing ClfA to add to plates coated with 5μg/ml fibrinogen. This was determined as $5x10^8$ c.f.u./ml and is the standard number of cells used in all solid phase adherence assays described subsequently

in this chapter. $5x10^8$ c.f.u./ml gave an approximate value of 0.350 on a spectrophotometer set to absorb at 600nm, as determined from a second standard curve (A_{600} vs bacterial cell number). This A_{600} reading was used to standardise the amount of cells used each time the assay was performed.

3.2.2. Screening for anti-Clf33(221-550) mAbs by indirect ELISA

The first successful hybridoma fusion was performed using Clf33(221-550) as an immunogen. This is the minimum truncate retaining fibrinogen-binding activity. Ten 96-well plates were screened by indirect ELISA for clones secreting mAbs reactive with immobilized Clf33(221-550). Approximately 50% of these wells contained viable clones. Initially, 130 clones which produced mAbs specific to Clf33(221-550) were transferred to 24-well plates. The clones that survived this initial transfer were then expanded to 10ml and subsequently 50ml flask stages of growth. Cell culture supernatants from each positive clone were screened by indirect ELISA after each transfer step. In the majority of cases clones were lost as a result of cell death, loss of mAb secretion or fungal contamination before cryopreservation.

3.2.3 Identification of anti-Clf33(221-550) mAbs which inhibited bacterial clumping in soluble fibrinogen and bacterial attachment to solid-phase fibrinogen.

When the 50ml flask stage of growth was achieved, it was found that eleven clones still secreted specific mAb at a high level. It was decided to purify antibody from all eleven clones by protein G-sepharose affinity chromatography (see section 2.6). This was necessary as the cell culture medium in which the Clf33(221-550) fusion was performed contained foetal bovine serum and hence fibrinogen. *S. aureus* Newman was capable of clumping in soluble bovine fibrinogen. It was important to remove all fibrinogen from the mAb preparations in order to assay the antibodies for inhibitory activity. Purified mAbs were subject to isotype determination and their concentration was measured (Table 3.1).

It was found that two out of the eleven clones assayed secreted mAbs that inhibited *S. aureus* Newman clumping in soluble human fibrinogen. One of these, mAb47, also demonstrated a weak ability to block bacterial attachment to solid-phase fibrinogen at a protein concentration of 453µg/ml. However, no activity was seen in

TABLE 3.1. Properties of monoclonal antibodies raised against Clf33 (221-550).

Clone	Isotype	Protein conc. (μg/ml)	Binding to Clf33(221-550)[a]	Binding to ClfA$^+$ bacteria: Clumping inhibition ID[b] (μg/ml)	Binding to ClfA$^+$ bacteria: Adherence inhibition ID (μg/ml)
17	IgG3	480	+	>480	>240.0
18	IgG2b	549	+	>549	>274.5
22	IgG2b	336	+	>336	>168.0
34	IgG2b	53	+	>53	>26.5
35	IgG1	906	+	>906	>453.0
36	IgG1	746	+	187	>373.0
43	IgG2a	613	+	>613	>306.5
47	IgG1	906	+	113	453.0[c]
48	IgG2a	816	+	>816	>408.0
54	IgG1	666	+	>666	>333.0
55	IgG1	853	+	>853	>426.5

[a] 10μg of purified mAb produced readings by indirect ELISA between 1.3 and 11.7 times the background.

[b] ID; Inhibitory Dose. The protein concentration where inhibition of bacterial interaction with fibrinogen was observed.

[c] mAb47 at this protein concentration produced 33% inhibition as compared to a positive control of uninhibited *S. aureus* cells.

this assay with mAb36 at 373μg/ml, the highest concentration tested in this assay. All purified mAbs from this fusion were subjected to isotype analysis. Out of the eleven mAbs characterized, five were of the IgG1 isotype, two were IgG2a, three were IgG2b and one was IgG3 (Table 3.1).

3.2.4 Screening for anti-Clf42(332-559) mAbs by indirect ELISA

This fusion was performed using Clf42(332-559) as the immunising antigen. Viable hybridoma clones were found in 261 out of 960 wells. However, this fusion was either quite unstable or else the cells grew more slowly in the fibrinogen-free medium relative to growth in medium containing 20% foetal bovine serum. The initial screening identified 60 clones which reacted with Clf42(332-559) on indirect ELISA. After expansion to the 50ml stage of growth, 38 reactive clones still were secreting high levels of mAb stably.

3.2.5 Identification of anti-Clf42(332-559) mAbs which inhibited bacterial clumping in soluble fibrinogen and bacterial attachment to solid-phase fibrinogen.

As this fusion was performed in fibrinogen-free medium, it was not necessary to affinity-purify the mAbs from the cell-culture medium in order to assay for blocking antibodies. Tissue culture supernatants from the 38 reactive clones grown to the 10ml flask stage were subject to ammonium sulphate precipitation. The antibody-containing precipitate was then resuspended in 1/10 of the original cell culture supernatant volume of PBS, thus concentrating the mAb approximately tenfold. These 1ml mAb preparations were then dialysed against PBS in order to remove residual ammonium sulphate which may have interfered with the inhibition assays.

Altogether, six mAbs were identified which demonstrated some form of inhibitory activity. Three of these mAbs interfered with bacterial clumping in soluble fibrinogen (Table 3.2). Also, all six showed varying degrees of ability to block adherence to solid-phase fibrinogen. Although some preliminary data was gathered from these clones, the only clone found to be stable enough to work with was that which secreted mAb6. The other five clones either stopped secreting mAb at an advanced stage of growth, or did not secrete antibody after thawing of cryopreserved material. The isotype of mAb6 was found to be IgG1.

TABLE 3.2. Properties of monoclonal antibodies raised against Clf42(332-559).

			Binding to ClfA$^+$ bacteria	
Clone	Isotype	Binding to Clf42(332-559) (A450)[a]	Clumping inhibition	adherence inhibition [b] (%)
3	IgM	0.794	+	75.45
6	IgG1	0.884	+	75.19
9	IgG1	0.910	AG[c]	59.43
21	IgG2a	1.048	+	41.86
22	IgG1	0.718	-	27.39
28	IgG1	0.916	-	35.82

[a] A450 readings produced on ELISA by 50µl of ammonium sulphate-precipitated mAb. Values shown are the mean of readings from duplicate wells.

[b] Figures are % inhibition compared to a control of *S. aureus* cells adhering to solid-phase fibrinogen.

[c] mAb9 caused aggregation of bacterial cells before addition of fibrinogen.

3.2.6 Specificity of anti-Clf33(221-550) polyclonal IgG and inhibitory mAbs for ClfA.

Anti-Clf33(221-550) polyclonal IgG, mAbs 36 and 47 raised against Clf33(221-550) and mAb6 raised against Clf42(332-559) were tested for the specificity of their interaction with ClfA by testing their ability to bind to the A domains of the Clf-Sdr multigene family of related SD repeat-containing proteins by ELISA. Data was expressed as % binding to Clf41(221-559) for mAbs 6, 36 and 47, and for anti-Clf33(221-550) polyclonal IgG. Binding of anti-ClfB(45-542) and anti-SdrD(50-566) polyclonal serum was expressed as a % binding to recombinant ClfB(45-542) and SdrD(50-566) respectively. These proteins share a low level of similarity (30%) to each other in discrete areas of region A (see introduction). Anti-Clf33(221-550) polyclonal IgG and mAbs 6, 36 and 47 bound only to recombinant Clf41(221-559) (Fig. 3.2). None of these antibodies showed reactivity with recombinant ClfB(45-542), SdrD(50-566) or SdrE(50-604). Also, specific polyclonal serum raised against ClfB(45-542) and SdrD(50-566) did not cross react with Clf41(221-559) in this assay. However, SdrD(50-566) polyclonal serum demonstrated some reactivity with recombinant SdrE(50-604).

3.2.7 Recognition of native ClfA on *S. aureus* cells by anti-Clf33(221-550) polyclonal IgG.

A qualitative colony dot-blot assay was used to determine if anti-Clf33(221-550) IgG was capable of specifically recognising ClfA present on the staphylococcal cell surface. This serum was tested for its reactivity with five different mutant strains of *S. aureus* (Fig 3.3). The strongest reaction was observed against the protein A deficient mutant NewmanΔ*spa*, and was slightly less against strain Newman that still expressed Spa. A NewmanΔ*spa*/*clfA*$^-$ double mutant, a Newman *clfA*$^-$ single mutant and a low ClfA expressing strain 8325-4 all showed an extremely low but detectable binding of the anti-Clf33(221-550) serum. No binding of preimmune serum from the same rabbit to any strain was observed. Unfortunately, binding of mAbs to the cell surface of staphylococcal cells was not detectable by this method. As mAbs would only recognise a single epitope rather than multiple epitopes over the whole surface of the protein, the signal was probably not sufficient to detect mAb binding using this particular method.

Figure 3.2. Specificity of anti-Clf33(221-550) polyclonal serum, anti-Clf33(221-550) mAbs 36 and 47, and anti-Clf42(332-559) mAb 6 for ClfA and related SD repeat-containing proteins. Data is shown as % binding to control Clf33(221-550) and Clf42(332-559) respectively and points are the mean of duplicate readings. Error bars show the standard deviation from the mean.

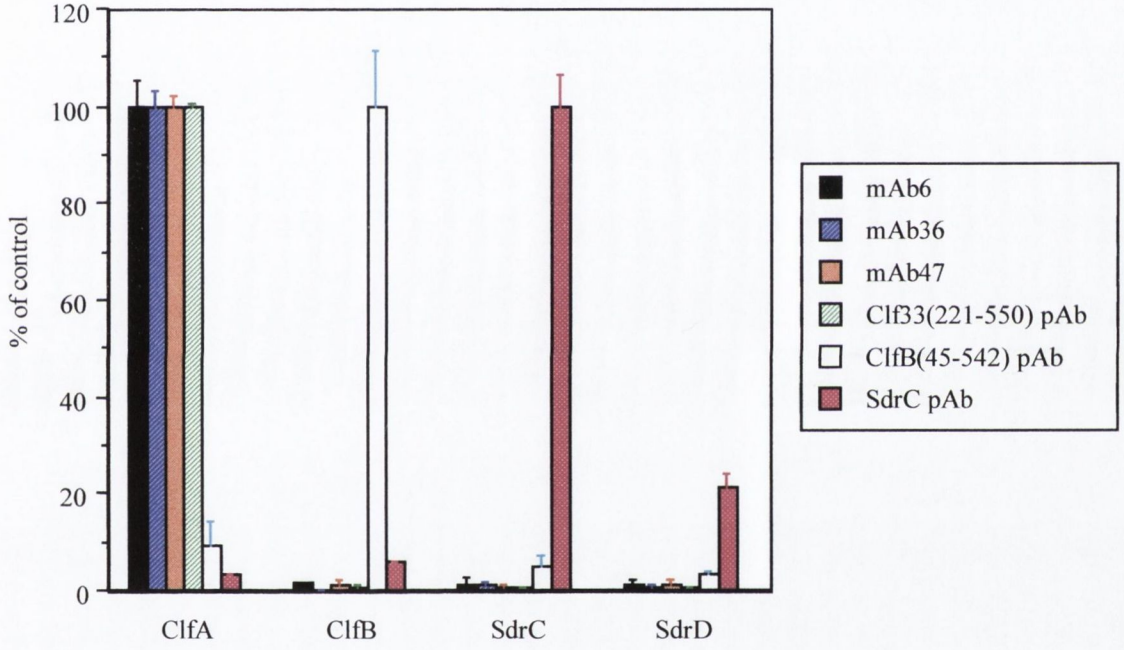
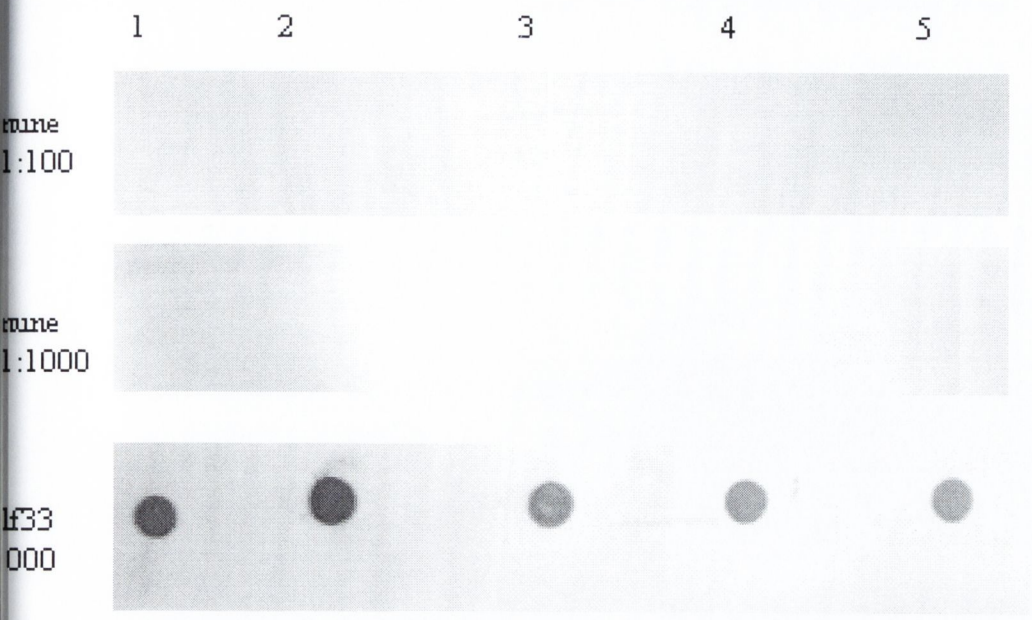

120
100
80
60
40
20
0
% of control
ClfA
ClfB
SdrC
SdrD
mAb6
mAb36
mAb47
Clf33(221-550) pAb
ClfB(45-542) pAb
SdrC pAb

Figure 3.3. Reactivity of anti-Clf33(221-550) polyclonal IgG with *S. aureus* whole cells immobilised on PVDF membrane. A, preimmune serum 1:100; B, preimmune serum 1:1000; C, anti-Clf33(221-550) IgG1:1000. Strain 1, Newman; 2, NewmanΔ*spa*; 3, Newman *clfA*$^{-}$; 4, NewmanΔ*spa*/*clfA*$^{-}$; 5, 8325-4. A description of these strains is given in Table 2.1.


1
2
3
4
5
rune
1:100
rune
1:1000
lf33
000

3.3 Discussion

From the panel of eleven mAbs identified against Clf33(221-550), the minimum fibrinogen-binding recombinant truncate, two were found to have the ability to block bacterial interactions with fibrinogen (Table 3.1). mAb47 showed inhibitory activity for bacterial clumping in soluble fibrinogen and bacterial adherence to solid-phase fibrinogen. The inhibitory activity of mAb36 was only detectable in the soluble assay. At the highest concentration tested in the solid-phase assay, no inhibition was observed. It is possible that mAb36 has a weaker affinity for ClfA, and that the clumping inhibition assay is more sensitive than the bacterial attachment assay. $2x10^7$ bacterial cells/120µl are used in the soluble fibrinogen-binding assay, where as $5x10^7$/100µl cells are required for the solid-phase attachment assay. This may be the reason why mAb36 activity is not detectable in the adherence-blocking assay. Also, there may be more than one epitope on ClfA to which antibody can bind in order to mediate a blocking effect. The epitope on ClfA recognised by mAb36 may be distinct from that bound by mAb47. The mAb47 epitope, when bound, may mediate a stronger inhibitory effect. The majority of antibodies isolated were IgG1 (5/11 from the Clf33(221-550) fusion and 4/6 from the Clf42(332-559) fusion). IgG1 is the most common IgG isotype at 65% of the total IgG (Jawetz, 1991).

Clf42(332-559), a truncate which does not bind fibrinogen, was used as an alternative to Clf33(221-550) as an antigen in this study for several reasons. Firstly, the possibility existed of mAbs against Clf33(221-550) were recognising Clf33(221-550)-fibrinogen complexes. Clf33(221-550) may have complexed with mouse fibrinogen after immunisation. As Clf42(332-559) does not bind to fibrinogen, but still elicits the production of blocking antibodies, it was a suitable alternative. If ClfA was to be used as a component in an anti-staphylococcal vaccine, a protein that bound to a host molecule such as human fibrinogen could not be considered. However, recent data suggests that this is not a concern for subcutaneous or intramuscular immunizations provided that no inflammation occurred (Patti, Personal communication). Also, there was concern that when screening for anti-Clf33(221-550) mAbs, Clf33(221-550) bound to ELISA wells could bind to the bovine fibrinogen present in the tissue culture medium. This may have identified mAbs recognising a Clf33(221-550)-bovine fibrinogen complex, which was not desired. This problem did not arise with the use of Clf42(332-559) due to its lack of ligand-binding activity.

Thirty eight mAbs were initially identified which reacted with Clf42(332-559), but only one clone, that which produced mAb6, was found to have suitable stability and produced a mAb with the required inhibitory properties (Table 3.2). It has been observed that somatic mutations can occur in hybridoma cells, the probability of which is increased by the number of cell divisions (Shacter, 1989). While rapid proliferation is useful for obtaining adequate numbers of cells, antibody production is not proportional to the rate of cell proliferation. Thus the monoclonality and indeed the antibody activity can be affected. Perhaps the Clf42(332-559) fusion cells divided with greater efficiency in serum-free medium, thus causing stability problems. Also, hybridoma cells are tetraploid, and this can create chromosomal instability. Perhaps hybridoma cell death occurred as a result of this.

The most potent mAb identified seems to be mAb47, although it is difficult to compare mAb6 directly with the other purified inhibitory mAbs as it is a crude preparation of unknown antibody concentration. The concentration of mAb in a hybridoma cell culture supernatant is approximately 50μg/ml (Harlow and Lane, 1988). Assuming no loss due to purification, the concentration of mAb6 in the preparation analysed in this chapter would be 500μg/ml at most, as the cell culture supernatant was concentrated tenfold by ammonium sulphate precipitation. No mAb was identified which blocked the ClfA-fibrinogen interaction completely. Only mAbs with weak partial inhibitory activity were found. Perhaps within the ClfA-fibrinogen blocking anti-Clf33(221-550) IgG it requires a combination of antibodies directed against several epitopes in order to mediate the potent inhibitory effect observed with the polyclonal antibody. Certainly in other systems, synergy using a combination of mAbs has been observed, where mAbs acting individually had a weak inhibitory effect (Shimonkevitz *et. al,* 1996, Lang *et. al.,* 1993).

Anti-Clf33(221-550) polyclonal IgG or any of the inhibitory mAbs identified in this chapter did not show any cross-reactivity with the A regions of related SD repeat-containing proteins by ELISA (Fig. 3.2). This demonstrates the specificity of the interaction between anti-Clf33(221-550) polyclonal IgG and mAbs 6, 36 and 47. The A domains of these proteins share 20-30% identical residues (see sections 1.4.8 and 1.4.9). Most of this similarity occurs in the region between ClfA residues 200-500. Both ClfA and ClfB posess a MIDAS motif within their respective region A domains. All proteins share a common five-residue motif with a consensus sequence of

TYTFTDYVD. The only other protein with significant similarity in the A region to ClfA is FnBPA (McDevitt *et. al.,* 1994). Unfortunately, this was not used as an antigen here as it has recently been discovered that the recombinant FnBPA region A binds to fibrinogen in vitro (Wann, Personal communication). Anti-Clf33(221-550) polyclonal IgG at a 1:1000 dilution is still weakly reactive with *S. aureus* cells carrying a mutation in ClfA and protein A (Fig. 3.3). As preimmune serum at a 1:100 concentration did not bind to the cells, the anti-Clf33(221-550) IgG is probably not binding to an IgG-binding protein, other than protein A, on the cell surface. The possibility exists that anti-Clf33(221-550) IgG cross reacts with another cell surface component of *S. aureus* other than the proteins used in the specificity ELISA.

CHAPTER 4

CHARACTERIZATION OF ANTI-CLUMPING FACTOR A MONOCLONAL ANTIBODIES INHIBITING THE CLUMPING FACTOR A-FIBRINOGEN INTERACTION.

4.1 Introduction

Antigen-specific antibodies, both polyclonal and monoclonal, are extremely useful tools for the study of the interactions of proteins with their corresponding ligands or substrates. This approach has been successfully used in the functional analysis of other bacterial proteins, including other secreted proteins and surface antigens of *S. aureus*. Monoclonal antibodies are particularly important as they may recognise functionally important epitopes on a molecule that interact with a substrate or ligand. Subsequent location of the epitope defined by mAbs that interfere with protein activity may yield an important insight into the mechanism of protein function. This is the approach taken in this study using mAbs found to be inhibitory to the ClfA-fibrinogen interaction.

4.1.1 Use of monoclonal antibodies in inhibition of bacterial protein activity.

This method has been successful in other studies using mAbs to dissect the pathogenic mechanisms of many virulent microorganisms and also in the characterization of the activity of bacterial proteins. For example, six different mAbs and a specific rabbit polyclonal antibody were raised against listeriolysin, the cytolysin of the pathogenic bacterium *Listeria monocytogenes* (Darji *et. al.,* 1996). The haemolytic activity of this toxin was prevented by polyclonal antibody as well as five of the mAbs. These mAbs localised to a region of the listeriolysin protein not previously implicated in mediating haemolytic activity, and therefore were useful in the discovery of this previously uncharacterized functionally important domain.

Also, specific mAbs produced against the toxic shock syndrome toxin of *S. aureus* (TSST-1) could either detect TSST-1 bound to the histocompatability locus antigen (HLA-DR) or inhibit the toxin association with HLA-DR (Shimonkevitz *et. al.,* 1996). One mAb which detected HLA-DR bound TSST-1 could also inhibit the toxin-induced activation of a T-cell receptor. Alternatively, mAbs specific for the HLA-DR association site could present TSST-1 in vitro, stimulating the proliferation of human T-cells. Therefore, the mAbs in this panel discriminated the superantigen functional sites on the TSST-1 molecule and demonstrated the property of being potent modulators of the toxic activity of the protein.

4.1.2 Inhibition of bacterial protein-ligand interactions using monoclonal antibodies

Many adhesin-ligand interactions have been characterized using mAbs, for example, the pathogenesis of the respiratory pathogen, *Bordetella pertussis.* The adherence of this organism to human respiratory cilia is critical to the pathogenesis of whooping cough. mAbs to eukaryotic glycoconjugates with specificity for substituted galactose-glucose-containing glycolipids blocked *B. pertussis* adherence when preincubated with ciliated epithelial cells (Tuomanen *et. al.,* 1988). Also, filamentous haemagglutinin (FHA), a 220-kDa protein located on the surface of *B. pertussis*, is one of the major cell adhesins and virulence factors of this bacterium. Three mAbs raised against this protein were found to have inhibitory activity (Leininger *et. al.,* 1993). All three mAbs seemed to be directed to the same epitope or to epitopes in close proximity as suggested by competition ELISAs. All mAbs were able to inhibit the adherence of Chinese hamster ovary cells to purified FHA. The attachment of *B. pertussis* to epithelial cell monolayers was inhibited by one of these mAbs. These antibodies proved to be very useful probes in the characterization of the functional domains of this important bacterial adhesin.

Virulence factors of other respiratory pathogens have also been characterized by use of mAbs. The adherence of pseudomonal species was investigated by using a newly developed microcolumn adherence assay (Mai *et. al.,* 1993). Mucoid strains of *Pseudomonas aeruginosa* were consistently more adherent than nonmucoid strains (30% versus 20%). mAbs directed against alginate were shown to inhibit the adherence of mucoid but not nonmucoid *P. aeruginosa.* In addition, synergism between combinations of mAbs and ciprofloxacin was also observed. These results indicated that bacterial alginate has an intrinsic role in the adherence of mucoid *P. aeruginosa* and may have evolved not only for protection against dehydration in the water and soil ecosystem of this bacterium, but also as a means of attaching to soil substrates in the same ecosystem in order to enhance survival. They also suggest that synergistic combinations of antibiotics with alginase or monoclonal antibodies to alginate may be of value in the therapy of some pseudomonal infections. Also, the attachment of *Mycoplasma bovis* to embryonic bovine lung cells was studied in order to identify factors participating in the adhesion process (Sachse *et. al.,* 1993). A mAb directed against a 26kDa protein of *M. bovis* was shown to reduce cytoadherence of

this organism. These findings demonstrated involvement of the 26kDa protein in *M. bovis* adherence.

It has been shown that polyclonal antibodies to *Borrelia burgdorferi* and also some mAbs to borrelia major surface proteins caused inhibition of adherence of the bacteria to cultured human umbilical vein endothelial cells. mAbs were generated for use in determining how *B. burgdorferi* outer surface protein A (OspA) is involved in mediating attachment to and invasion of, endothelial cells by this pathogen (Comstock *et. al.,* 1993) Fab molecules from one anti-OspA MAb, 9B3D, demonstrated an inhibitory effect on bacterial association with endothelial cells. Immunoblots of *E. coli* expressing different OspA fusion peptides suggested that the 9B3D epitope is located in the carboxy-terminal half of OspA. mAb 9B3D is therefore a valuable tool for elucidating the domain or domains of OspA involved in the endothelial cell adherence of *B. burgdorferi.*

An approach using mAbs has also been used in the characterization of several oral microorganisms and their adherence to components of the mouth cavity. A mAb to *Actinomyces naeslundii* type 1 fimbriae was capable of inhibiting the adherence of this bacterium to salivary proline-rich protein-treated hydroxyapatite (Nesbitt *et. al.,* 1996). This IgG mAb, 86-49E, blocked the adsorption of the parent strain to proline-rich protein-treated hydroxyapatite by 77% with 1.0 μg/ml of the monoclonal antibody. Immunogold labelling in conjunction with electron microscopy suggested binding of mAb 86-49E occurring near the distal end of the fimbriae. These data suggested that this mAb against type 1 fimbriae of *A. naeslundii* that is capable of binding to a discrete site on the fimbriae has the capacity to inhibit the adsorption of this organism to saliva-treated hydroxyapatite. Oral streptococci can also be involved in the pathogenesis of dental caries. Analysis has indicated indicated that a 19-amino-acid peptide sequence (residues 435 to 453 of GtfC) within a highly conserved region of the glucosyltransferases of the cariogenic streptococci might be functionally important. The concentrated hybridoma supernatant of six reactive mAbs inhibited GtfC enzymatic activity but failed to inhibit GtfD, although GtfD contains the same peptide sequence, demonstrating high specificity (Chia *et. al.,* 1993). Furthermore, the in vitro sucrose-dependent adherence of *Streptococcus mutans* was inhibited by the same antibody. These results indicate that the peptide containing the N-terminal conserved region of

glucosyltransferases is functionally important for both enzymatic activity and bacterial adherence.

Attachment of *S. mutans* to the tooth surface also involves a cell surface protein with an M_r of 185kDa, termed streptococcal antigen (SA) I/II. Four overlapping fragments of the gene encoding SA I/II were amplified by polymerase chain reaction, cloned, and expressed in *E. coli* (Munro *et. al.,* 1993). The recombinant polypeptides were assayed for adhesion-binding activity to salivary receptors and for recognition by a panel of mAbs raised against SA I/II. Two of the mAbs that are known to prevent colonization of *S. mutans* in vivo bound the recombinant polypeptide comprising residues 816 to 1161. The fact that the mAbs prevented colonization of *S. mutans* suggests that an adhesin-binding activity resides within the portion of SA I/II comprising residues 816 to 1213, which is highly conserved among oral streptococcal species.

4.1.3 Use of antibodies in the functional analysis of staphylococcal adhesins.

4.1.3.1 Collagen binding protein.

Polyclonal antibodies raised against a recombinant form of the collagen adhesin inhibited the binding of ^{125}I-labelled type II collagen to *S. aureus* cells (Patti *et. al.,* 1995). Polyclonal antibodies raised against the purified adhesin also inhibited bacterial attachment to cartilage (Switalski *et. al.,* 1993). Also, mice actively immunised with the recombinant collagen-binding domain were protected against sepsis-induced death (Nilsson *et. al.,* 1998). This protection could also be conferred by passive transfer of immune serum. This immune serum also promoted opsonophagocytosis and intracellular killing of *S. aureus* cells, indicating that the immune response against this molecule may be protective against staphylococcal infection. However, specific identification of immunologically relevant epitopes has not been performed at the molecular level.

4.1.3.2 Fibronectin binding proteins.

It has proved extremely difficult to generate antibodies that block the interaction between the fibronectin-binding proteins and fibronectin. Recently, motifs within the D repeats of FnBPA which stimulate the production of blocking antibodies

were identified (Sun *et. al.,* 1997). FnBPA possesses three 37- or 38-residue motifs (D1, D2 and D3) that can each bind fibronectin with low affinity. In tandem, these repeats have a high binding affinity for fibronectin. Polyclonal antibodies generated against a GSTD1-3 fusion protein had the ability to inhibit fibronectin binding to *S. aureus*. However, antibody concentrations in excess of 20μg/ml did not promote more than 50% inhibition. Antipeptide antibodies to D3 epitopes also achieved a partial blocking effect.

Monoclonal antibodies have been identified which markedly enhance the binding of fibronectin to recombinant *Streptococcus dysgalactiae* FnbA fragments (Speziale *et. al.,* 1996). It was proposed that these enhancing mAbs changed the unordered structure of the unoccupied ligand-binding unit to a conformation normally induced by fibronectin binding. Pure mAb did not recognise the antigen by itself, and the presence of fibronectin was necessary for the immunological interaction between the antibody and the epitope. The epitope was located to a region just N-terminal of the ligand binding site. These mAbs probably change the unordered structure of an unoccupied ligand-binding unit to a conformation normally induced by fibronectin binding. It is known that this recombinant FnBP fragment has little or no secondary structure (House-Pompeo *et. al.,* 1996). Upon ligand binding, the secondary structure of this domain becomes predominantly β-sheet, as determined by circular dichroism spectroscopy studies. These ligand-induced binding site mAbs occur in human convalescent patient serum (Casolini *et. al.,* 1998). It seems that this protein has evolved a mechanism to avoid the production of blocking antibody by the host. Bacteria can still adhere to immobilized fibronectin or bind soluble fibronectin even in the presence of an immune response.

4.1.3.3 Laminin binding protein.

Monoclonal antibodies have been isolated against *S. aureus* laminin-binding proteins (Mota *et. al.,* 1988). Five stable cells lines were isolated, one of which, an IgM antibody, blocked the binding of laminin to bacteria and recognised the 52kDa receptor on whole bacterial extracts. This mAb and also four other non-inhibitory mAbs bound to the 67kDa laminin-binding protein from mouse melanoma cells. The inhibitory mAb also reacted by immunoblot analysis with gp43 from the fungus *Paracoccidioides brasiliensis*, also a laminin receptor (Vincenti *et. al.,* 1997). This

mAb could also inhibit the laminin-mediated adhesion of *P. brasiliensis* to epithelial cells as well as the infection caused by this organism in vivo. The fact that a mAb raised against the laminin-binding protein from one organism can block adherence by a receptor from another suggests that there may be evolutionary conservation of epitopes important in laminin recognition. However, the putative laminin-binding protein of *S. aureus* has not been defined at the molecular level.

4.1.4 Objective of this study.

This chapter deals with the characterization of the fibrinogen-binding activity of ClfA using both polyclonal and monoclonal antibodies. The inhibition of *S. aureus* clumping in soluble fibrinogen by anti-Clf33(221-550) polyclonal IgG, anti-Clf33(221-550) mAbs 36 and 47, and by anti-Clf42(332-559) mAb6 as well as the dose-dependent inhibition by polyclonal IgG and the mAbs of bacterial binding to immobilized fibrinogen is studied. Also, the effect of polyclonal IgG and mAbs on the binding of fibrinogen to solid-phase Clf41(221-559) is investigated.

4.2 Results

4.2.1 Inhibition of *S. aureus* clumping in soluble fibrinogen.

Anti-Clf33(221-550) purified polyclonal IgG is a very potent inhibitor of *S. aureus* clumping in soluble fibrinogen, with an inhibitory dose of 20μg/ml (Table 4.1). Preimmune rabbit serum at a 1:2 dilution did not inhibit the clumping of bacterial cells. Each of the IgG purified mAbs assayed individually blocked clumping weakly with inhibitory doses in the range 125 to 250μg/ml. No non-specific aggregation of cells in the presence of antibody before the addition of fibrinogen was observed. An isotype-matched negative control antibody, mAb35, did not inhibit clumping at the highest concentration tested in this assay. When assayed in combination, mAbs 6+36 and 6+47 did not demonstrate any improved activity much beyond their individual inhibitory doses (Table 4.2). Both of these combinations yielded an endpoint of 125μg/ml. However, mAbs 36+47 had a potent combined inhibitory effect. Inhibition occurred at a concentration of 31.25μg/ml.

TABLE 4.1. Inhibition of *S. aureus* clumping in soluble fibrinogen by individual anti-ClfA mAbs. Data shown is representative of two separate assays.

antibody	inhibitory dose[a] (μg/ml)
polyclonal IgG	20
mAb6	125
mAb36	250
mAb47	125
mAb35	>1000

[a] Inhibitory dose is the final concentration of antibody where clumping of bacterial cells is prevented.

TABLE 4.2. Inhibition of *S. aureus* clumping in soluble fibrinogen by paired combinations of anti-ClfA mAbs. Data shown is representative of two individual experiments.

mAb combination	inhibitory dose (μg/ml)
6+36	125.00
6+47	125.00
36+47	31.25

4.2.2 Inhibition of bacterial attachment to solid-phase fibrinogen.

Anti-Clf33(221-550) polyclonal IgG blocked bacterial attachment to solid-phase fibrinogen in a dose-dependent manner (Fig. 4.1). 50% inhibition occurred at a dilution of 1:4000 that corresponded to a protein concentration of approximately 1.5μg/ml. Preimmune rabbit IgG did not inhibit bacterial attachment at 1:100 dilution. These results suggest that anti-Clf33(221-550) polyclonal IgG recognised epitopes conformationally linked to or directly at the active site of ClfA, thereby directly inhibiting fibrinogen binding.

Although mAb36 inhibited clumping in the soluble fibrinogen assay, bacterial adherence could not be inhibited in this assay at a final concentration of 1000μg/ml (Fig. 4.2). No significant inhibition was shown by mAb6 at this protein concentration. However, mAb47 was the only mAb found to have a significant amount of blocking activity individually. This occurred in a dose-dependent manner, with 50% inhibition occurring at approximately 800μg/ml. An isotype-matched negative control antibody, mAb35 (Table 4.1) had no effect at the highest concentration used.

The mAbs were assayed in paired combinations as for the clumping inhibition assay. mAbs 6+36 together had very little effect (Fig. 4.3). When mAbs 6+47 were used in combination, an unusual but repeatable pattern was seen. At protein concentrations between 1000 and 4μg/ml, an approximate 20% enhancement of bacterial attachment to the wells was observed. Again, mAbs 36+47 were potent inhibitors of *S. aureus* adherence in a dose-dependent manner. 50% inhibition occurred at approximately 16μg/ml. Also, the combined cell culture supernatants from mAbs 36 and 47 inhibited bacterial attachment to fibrinogen by 80% (Fig. 4.4).

4.2.3 Inhibition of fibrinogen binding to solid-phase Clf41(221-559).

In this system as in the bacterial adherence assay, anti-Clf33(221-550) polyclonal IgG inhibited purified fibrinogen binding to Clf41(221-559) in a dose-dependent manner (Fig. 4.5). The ID_{50} occurred at a dilution of 1:12000, corresponding approximately to a protein concentration of 0.5μg/ml. This suggests that this assay is three-fold more sensitive than bacterial adherence, where the ID_{50} is 1.5μg/ml. However, inhibition was incomplete, as at 1:200, the highest dilution of antibody tested, 20% binding of fibrinogen to Clf41(221-559) still occurred.

Figure 4.1 Inhibition of bacterial attachment to 5μg/ml solid-phase fibrinogen by anti-Clf33(221-550) polyclonal IgG. Results are expressed as a % of *S. aureus* cells attaching to solid-phase fibrinogen in the absence of inhibiting antibodies. Data shown is taken from one representative experiment. Points are the mean of triplicate ELISA wells and error bars are the standard deviation from the mean.

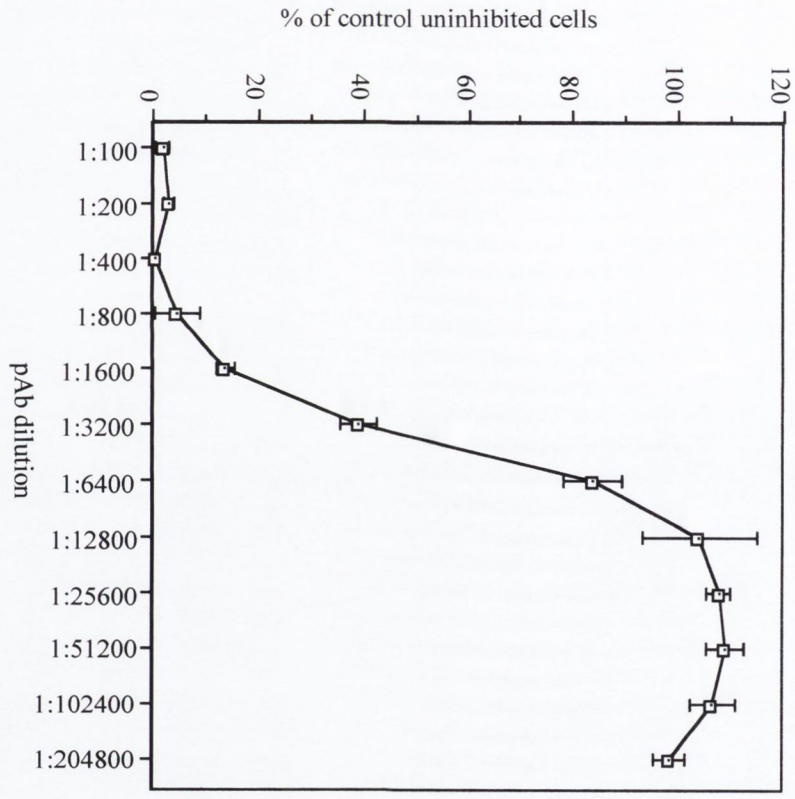

% of control uninhibited cells
0
20
40
60
80
100
120
pAb dilution
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
1:102400
1:204800

Figure 4.2. Inhibition of bacterial attachment to solid-phase fibrinogen by anti-Clf33(221-550) mAbs 36 and 47 and anti-Clf42(332-559) mAb 6. Results shown are taken from one representative experiment. Data points are the mean of triplicate determinations and error bars show the standard deviation from the mean.

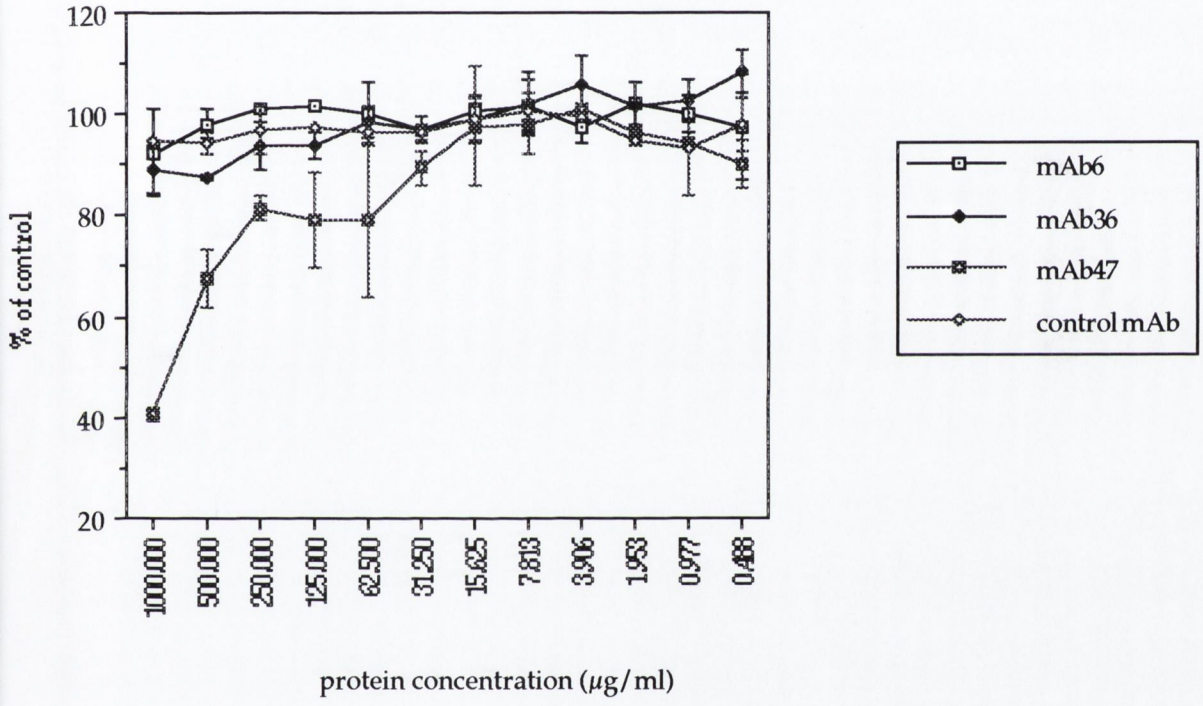

120
100
80
60
40
20
% of control
1000.000
500.000
250.000
125.000
62.500
31.250
15.625
7.813
3.906
1.953
0.977
0.488
protein concentration (µg/ml)
mAb6
mAb36
mAb47
control mAb

Figure 4.3. Inhibition of bacterial attachment to solid-phase fibrinogen by paired combinations of anti-ClfA mAbs 6, 36 and 47. Data is taken from one representative experiment. Point values shown are the mean of triplicate ELISA wells. Error bars show the standard deviation from the mean.

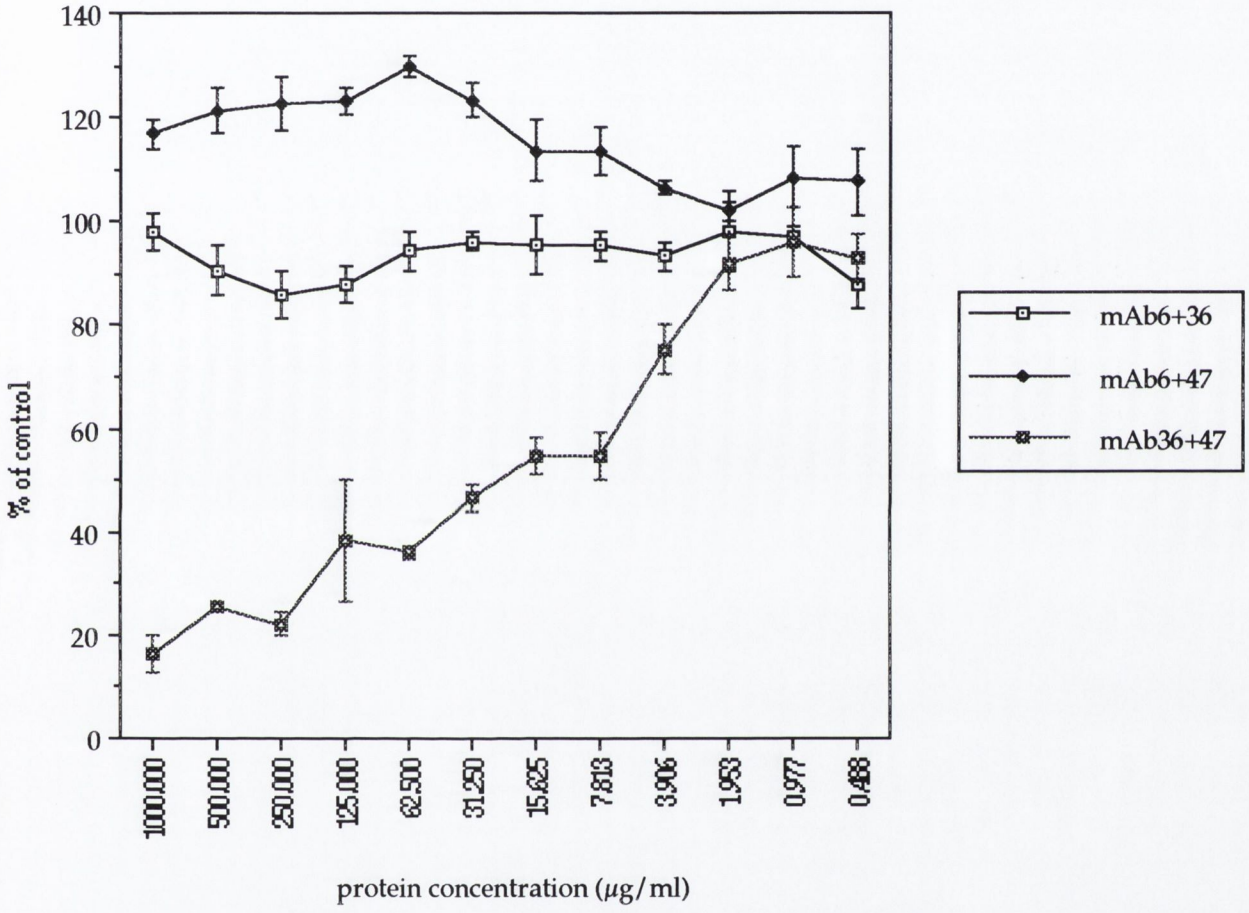

140
120
100
80
60
40
20
0
% of control
1000.000
500.000
250.000
125.000
62.500
31.250
15.625
7.813
3.906
1.953
0.977
0.488
protein concentration (μg/ml)
mAb6+36
mAb6+47
mAb36+47

Figure 4.4. Inhibition of bacterial binding to solid-phase fibrinogen by cell culture supernatants containing anti-ClfA mAbs. Typical mAb concentrations in cell culture supernatants are in the range 10-50μg/ml. Data points are the mean of triplicate wells from one representative ELISA. Error bars show the standard deviation from the mean.

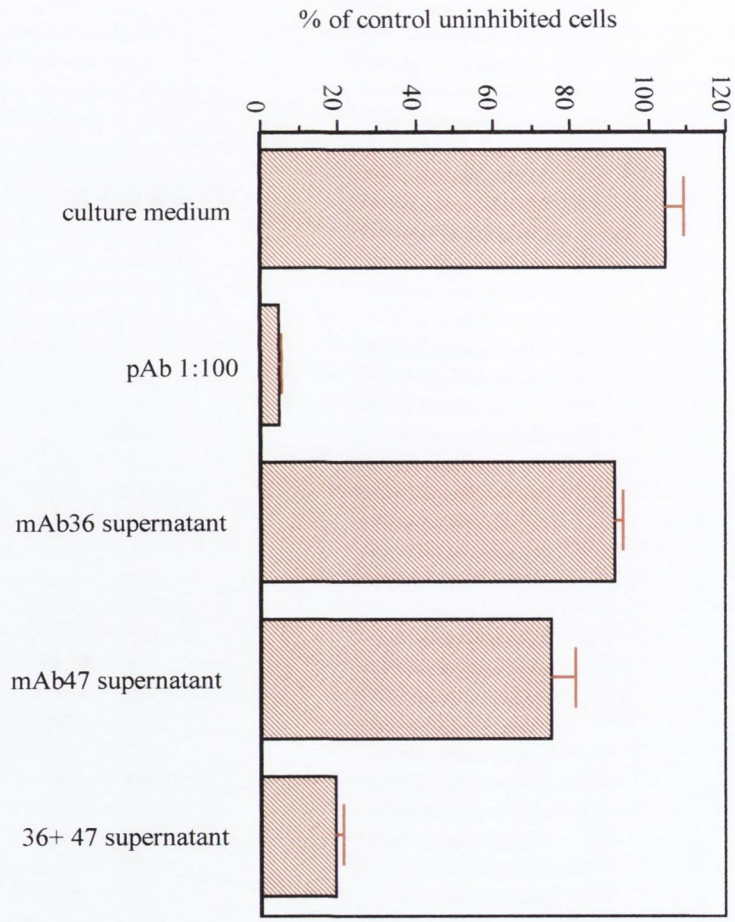
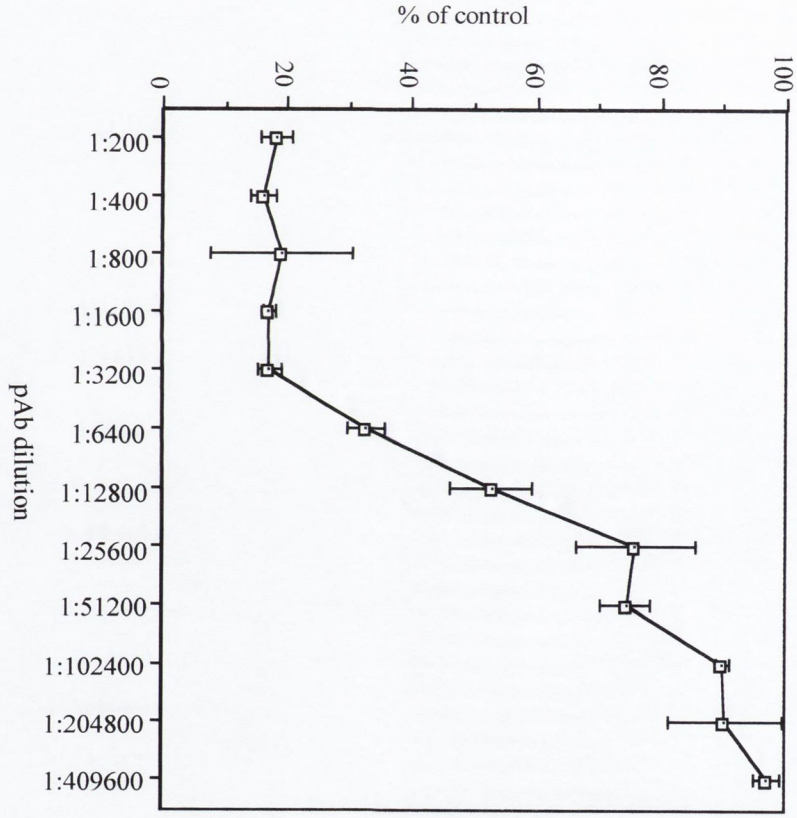

% of control uninhibited cells
0
20
40
60
80
100
120
culture medium
pAb 1:100
mAb36 supernatant
mAb47 supernatant
36+ 47 supernatant

Figure 4.5. Inhibition of fibrinogen binding to immobilized Clf41(221-559) by anti-Clf33(221-550) polyclonal IgG. Data shown is representative of one experiment, and points are the mean of triplicate ELISA wells. Error bars represent the standard deviation from the mean.


% of control
0
20
40
60
80
100
pAb dilution
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
1:102400
1:204800
1:409600

Because of the increased sensitivity of this system, inhibitory activity of individual mAbs was much more readily demonstrated (Fig. 4.6). mAbs 6 and 36 both showed a dose-response effect. However, the inhibitory effect seen by both of these mAbs was still quite weak. 75% of control fibrinogen binding occurred at final IgG concentrations of 62 and 15μg/ml for mAbs 6 and 36 respectively. In contrast, mAb47 activity was more pronounced. A concentration of 10μg/ml of this mAb was capable of producing 50% inhibition of fibrinogen binding to Clf41(221-559). A dose-response effect was also seen. Again, the negative control IgG1 mAb showed no effect. When assayed in combination, mAbs 36+47 could not inhibit fibrinogen binding beyond 50%, and did not demonstrate synergy in this assay (Fig. 4.7). Also, no synergism was observed using mAbs 6+36 or 6+47 together.

4.3 Discussion

Anti-Clf33(221-550) polyclonal antibodies have been found to be a potent inhibitor of *S. aureus* clumping in soluble fibrinogen. This anti-Clf33(221-550) polyclonal IgG was used as a positive control for inhibition in all assays using the mAbs. Anti-Clf33(221-550) polyclonal IgG was also found to be extremely effective in preventing *S. aureus* cells binding to solid-phase fibrinogen. Previously, purified IgG generated against ClfA full-length region A was a potent inhibitor of adherence of radiolabelled *S. aureus* to fibrinogen-coated coverslips (McDevitt *et. al.,* 1995). Although the potent polyclonal IgG provided a positive inhibition control for the assays using the mAbs, it was difficult to compare directly the effect of polyclonal IgG with mAbs in this study due to its heterogeneous nature compared to the monospecific activity of the mAbs.

No mAb with a similar level of potent inhibitory properties was identified, either in the soluble fibrinogen clumping assay or the solid-phase adherence assay. Each of the three blocking mAbs characterized had a weak effect individually. In both the soluble clumping assay and the bacterial adherence assay, mAbs 36 and 47 in combination show a synergistic inhibitory effect on bacterial interactions with fibrinogen. These mAbs may bind to important epitopes, either distinct from each other or overlapping in nature. Effects are seen in combination at quite low protein concentrations for both assays involving whole cells in this section of the study. The

Figure 4.6. Inhibition of fibrinogen binding to solid-phase Clf41(221-559) by anti-ClfA mAbs. The control mAb (mAb35) is a non-inhibitory IgG1 raised against Clf33(221-550). One representative set of data is shown. Data points are the mean of triplicate ELISA wells. Error bars are the standard deviation from the mean.

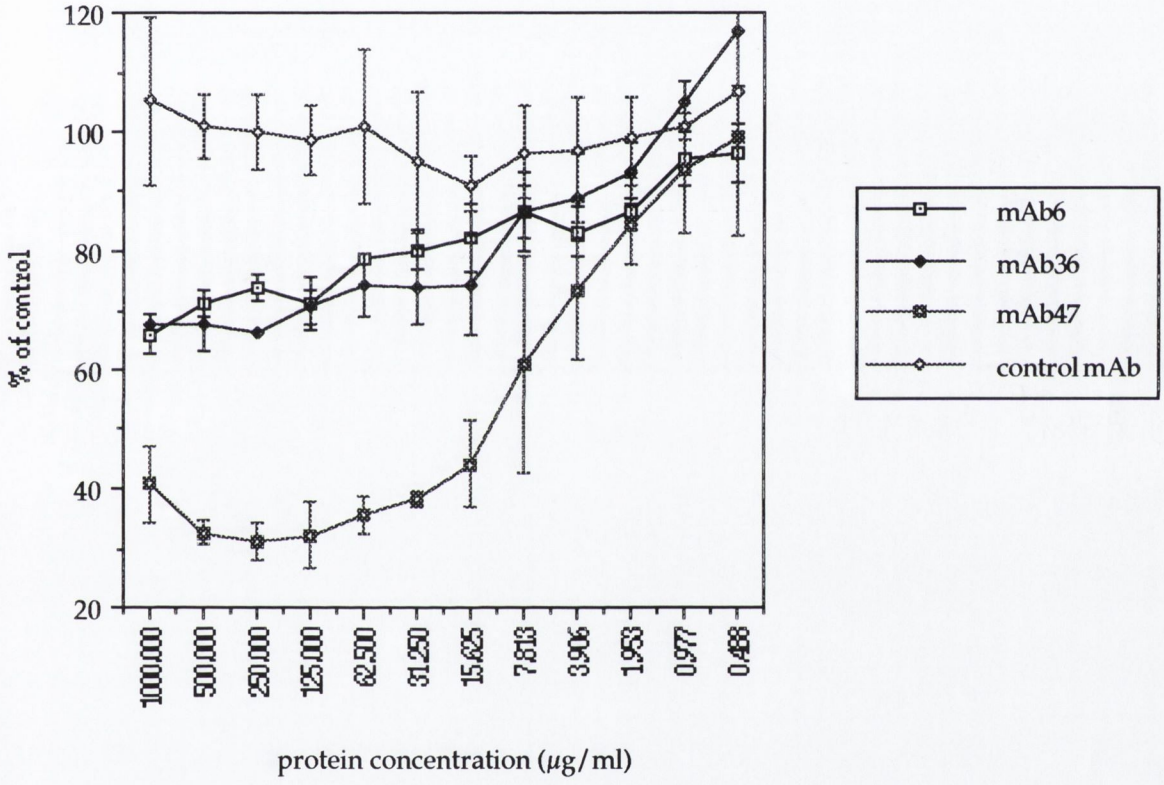
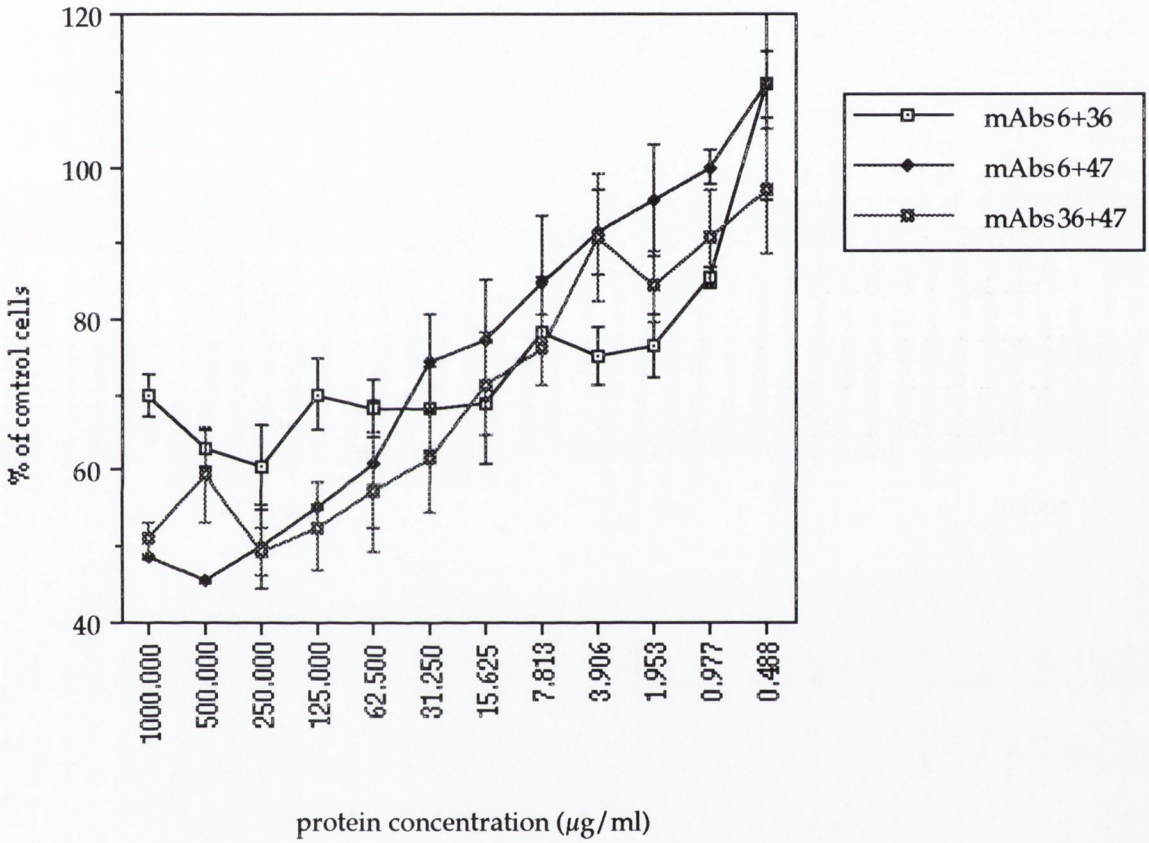

120
100
80
60
40
20
% of control
1000.000
500.000
250.000
125.000
62.500
31.250
15.625
7.813
3.906
1.953
0.977
0.488
protein concentration (μg/ml)
mAb6
mAb36
mAb47
control mAb

Figure 4.7. Inhibition of fibrinogen binding to immobilised Clf41(221-559) by paired combinations of anti-ClfA mAbs. One representative experiment is shown and points are the mean of triplicate ELISA wells. Error bars show the standard deviation from the mean.


120
100
80
60
40
% of control cells
1000.000
500.000
250.000
125.000
62.500
31.250
15.625
7.813
3.906
1.953
0.977
0.488
protein concentration (µg/ml)
mAbs6+36
mAbs6+47
mAbs36+47

combined tissue culture supernatants from the hybridoma clones secreting both mAbs could also inhibit bacterial adherence effectively. A synergistic effect as a result of combining mAbs has been shown in other systems. For example, two mAbs produced against TSST-1 of *S. aureus*, 327H and 769U, could inhibit the association of TSST-1 with its ligand HLA-DR by approximately 32 and 84% respectively, at IgG concentrations of 20μg/ml (Shimonkevitz *et. al.,* 1996). Also, 10μg/ml 769U in combination with a suboptimal amount (also 10μg/ml) of 710U, 327H or 49A resulted in greater than additive inhibitions. The best example of this was using 769U and 327H together. This combination of mAbs produced 80% inhibition of TSST-1 binding to Raji cells. The synergistic effect on the interaction of TSST-1 with HLA-DR was observed only with combinations containing the most potent mAb, 769U. Other mAb combinations inhibited at additive levels, which suggested that 769U recognised a TSST-1 epitope distinct from the other four mAbs tested.

It is evident that the inhibition mediated by anti-Clf33(221-550) polyclonal antibodies must be as a result of antibody recognition of a combination of functionally crucial ClfA epitopes. mAbs 36 and 47 may recognise two of these important domains, but antibodies directed against further epitopes may also be required in order to mediate the more potent effect seen with polyclonal IgG. Also, the polyclonal antibodies were raised in a rabbit, while the monoclonals were produced using immune murine splenocytes. The immune response against Clf33(221-550) was probably not identical in both species, and may indeed have been very different. The difference in species reactivity to the antigen may account for weaker inhibitory antibodies generated in the mouse. Due to the difficulties in both generating sufficient mouse polyclonal IgG or indeed making rabbit mAbs, unfortunately it could not be shown whether the difference in species-specific immune response to ClfA is important in this study.

No synergistic inhibitory effect is seen with mAbs 6+36 or 6+47 in combination in the clumping assay or the bacterial adherence assay. However, mAbs 6+47 combined demonstrated an unusual but repeatable enhancement effect in the bacterial adherence assay. It has been shown that the ClfA native receptor on the cell surface is highly conformational in nature. The Clf33(221-550) truncate has been found to exist in solution in an ordered conformation relative to the full length region A construct, Clf40(40-559). Also, a recombinant protein composed of region A and region R showed an extremely unordered loosely folded conformation in solution, suggesting

that the native receptor may exist in this state on the bacterial cell surface. (Nanavaty, Personal communication). The action of mAbs 6+47 may act together on the native receptor to expose the ClfA fibrinogen-binding sites more readily and thus increase the fibrinogen binding ability. This combination of mAbs may have caused aggregation of bacterial cells at high protein concentration, and this may give a false impression of increased adherence in this assay. However, no obvious aggregation of bacterial cells in the presence of mAbs 6 and 36 combined was observed. It is still possible a low level of bacterial agglutination was occurring at a microscopic level. Unfortunately, this was not tested when these assays were being performed.

Distinct epitopes are recognised by mAbs 6 and 47. This is enhanced by the fact that a dramatic increase in mAb6 binding to the Clf41(221-559)-fibrinogen complex was also seen (see section 6.2.6). Fibrinogen binding to Clf41(221-559) seems to increase exposure of epitope for mAb6. This enhancement effect mediated by mAbs 6 and 47 combined was not seen in the solid-phase Clf41(221-559) binding ELISA. As already mentioned, Clf41(221-559) when bound to plastic may be conformationally constrained, and these two mAbs combined may not be able to mediate the same effect as seen with the whole cell adherence assay. This suggests that the enhancement effect seen may be caused by a conformational effect of mAbs 6 and 47 in combination on the native receptor on the cell surface of *S. aureus*. mAbs have been produced which recognise epitopes within the fibronectin-binding domain of the FnbA (fibronectin receptor) of *Streptococcus dysgalactiae* which markedly enhanced rather than inhibited binding of fibronectin to recombinant FnbA and whole bacterial cells (Speziale *et. al.,* 1996). It was proposed that these enhancing mAbs changed the unordered structure of an unoccupied ligand binding unit to a conformation normally induced by fibronectin binding. A similar phenomenon may be occurring with mAbs 6 and 47 combined in the bacterial adherence assay. The N-terminus of region A may play an important role in the overall conformation of the native molecule, although it is not directly involved in fibrinogen binding. A difference in Ca^{2+} inhibition kinetics is seen for Clf40(40-559) and Clf41(221-559) (O'Connell *et. al.,* 1998). Unfortunately, full-length region A could not be used in these experiments. It is impractical to work with as it rapidly breaks down into the shorter form, corresponding to Clf33(221-550) or Clf41(221-559) truncates.

As already mentioned, the synergistic effect of mAbs 36 and 47 combined may occur as a result of the mAbs recognising overlapping epitopes. However, it is clear that mAb36 is not as potent in inhibiting the ClfA-fibrinogen interaction as mAb47, and it also must recognise a separate epitope, although this may overlap that defined by mAb47. This is possibly why the same enhancement effect does not occur with mAb6 and 36 combined.

Anti-Clf33(221-550) polyclonal IgG was also found to inhibit the binding of fibrinogen to polystyrene-bound Clf41(221-559) in a dose-responsive manner. However, at the highest dilution tested, no more than 80% inhibition of fibrinogen binding to solid-phase Clf41(221-559) could be achieved. Evidence from other assays suggests that Clf41(221-559) bound to the solid-phase may be conformationally constrained (see section 6.2.6). A more pronounced inhibitory effect was seen for mAb47 when the inhibition of fibrinogen binding to solid-phase Clf41(221-559) was studied, and an effect of mAbs 6 and 36 could also be seen here, unlike in the bacterial adherence assay. This is probably due to the improved sensitivity of using an ELISA based on purified protein as compared to a whole cell assay. Data for the anti-Clf33(221-550) polyclonal IgG suggests that the Clf41(221-559) solid-phase assay is approximately three-fold more sensitive than the bacterial adherence assay. This was assumed on comparison of ID_{50} data for both assays, 0.5μg/ml and 1.5μg/ml respectively. Also, the epitopes defined by the mAbs could be more available in the absence of residues 40-220 of region A.

The bacterial adherence and the Clf41(221-559) solid-phase ELISA data suggests that mAb47 may be slightly more potent when assayed individually. No inhibitory effect of mAbs 6 and 36 was seen in the whole cell assay even when used at a high protein concentration of 1000μg/ml. This assay is limited with respect to sensitivity, as a relatively large number of cells must be used in order to obtain a positive reading. Using Scatchard analysis, another study has estimated that ClfA-expressing *S. aureus* Newman cells bound approximately 2130 fibrinogen molecules per bacterial cell, with an estimated dissociation constant of 9.9nM. In order to inhibit this interaction, at least the same number of antibody molecules would be required in order to saturate the ClfA receptors, and possess a similar affinity to fibrinogen as that of ClfA. Quite a large amount of mAb would be required, especially if a given antibody does not mediate a potent inhibitory effect, either due to low affinity or non-recognition

of vital inhibitory epitopes. Partial blocking by mAbs is a common phenomenon. One example is in the presence of 50μg/ml mAb which recognised laminin binding epitope only a 50% reduction in adherence to epithelial cell monolayers could be achieved (Vincenti *et. al.,* 1997). Also, between 2- and 15-fold molar excess of mAbs raised against listeriolysin of *L. monocytogenes* was necessary to obtain 50% toxin neutralisation, possibly reflecting the differences in the affinities of the mAbs (Darji *et. al.,* 1996).

In the solid-phase Clf41(221-559) binding assay, the binding of fibrinogen to Clf41 could not be inhibited by mAbs 36+47 combined more than 50%. This may be due to the fact that the Clf41(221-559) recombinant truncate is less conformationally flexible than the native ClfA protein on the bacterial cell surface. Circular dichroism analysis of ClfA recombinant truncates has shown that full length region A has a greater ability to fold in solution than the shorter fibrinogen-binding truncates Clf33(221-550) and Clf41(221-559) (Nanavaty, Personal communication). Also, when Clf41(221-559) is bound to a polystyrene surface, it has an extremely limited capability to fold (see section 6.2.6). However, anti-Clf33(221-550) polyclonal serum can only block fibrinogen binding to Clf41(221-559) in this assay by a maximum of 80% (Fig. 4.5). This demonstrates that Clf41(221-559) bound to polystyrene is more conformationally constrained than native ClfA free to fold in solution. It is also clear from the clumping and adherence data presented here that these inhibitory mAbs are capable of recognising blocking epitope(s) on native ClfA which are accessible to the mAbs when the protein is expressed on the cell surface of *S. aureus*.

CHAPTER 5

ADHERENCE OF *S. AUREUS* TO PLATELET-FIBRIN CLOTS IN VITRO AND VIRULENCE MODELS IN VIVO

5.1 Introduction

5.1.1 Role of *S. aureus* in the pathogenesis of infectious endocarditis

S. aureus now causes 25 to 35% of endocarditis cases (Sanabria *et. al.,* 1990, Sandre and Shafran, 1996). This disease is most common in intravenous drug users, elderly patients, people with prosthetic devices and hospitalized patients. *S. aureus* is one of the most frequent causes of nosocomial and prosthetic valve endocarditis, and intravenous catheters are the most frequent source of bacterial inoculation. The mortality rate for nosocomial endocarditis is 40 to 56%, and is even higher when the pathogen is *S. aureus* (Fernandez-Guerrero *et. al.,* 1995). Bacteria which cause infective endocarditis have the capacity to adhere to damaged cardiac tissue and to platelet-fibrin clots in vitro (Baddour, 1994, Crawford and Russell, 1986, Gould *et. al,* 1975, Heraief *et. al.,* 1982). Therefore, a likely mechanism for the initiation of the infection process is the direct attachment of bacteria in the bloodstream to damaged heart valves (Baddour, 1994, Angrist and Oka, 1963). This involves a series of complex interactions between the bacteria and several host components. Numerous factors associated with damaged or normal cardiac endothelium can mediate bacterial adherence in vitro such as platelets, von Willebrand factor, fibrinogen, fibronectin, and the endothelium layer itself (Hamill, 1987).

The platelet-fibrin clot adherence model described previously (Moreillon *et. al.,* 1995, Rampyari *et. al.,* 1990, Toy *et. al.,* 1985) provides a more realistic insight into bacterial adherence in vivo than the study of adherence to highly purified fibrinogen in vitro. Several bacterial cell surface components were shown to be involved in interactions with components of cardiac vegetations (Baddour, 1994). These include several surface-located proteins of staphylococci that promote attachment to purified host components in vitro and possibly capsular polysaccharide in a masking capacity (Cheung *et. al.,* 1992, Cheung *et. al.,* 1994, Kuypers and Proctor, 1989).

5.1.2 *S. aureus* surface components involved in adherence to cardiac vegetations

5.1.2.1 Capsular polysaccharide

S. aureus posesses several surface determinants which could potentially mediate adherence to damaged heart valves and promote endocarditis. One of these may be capsular polysaccharide, although it has proved difficult to demonstrate a definite role for the capsule in this type of infection. In the rat model of experimental endocarditis,

antibodies to capsular polysaccharide were not protective against *S. aureus* endocarditis (Lee and Nemeth, 1995). Similar data was obtained in a rabbit endocarditis model, where whole-cell induced *S. aureus* antibody did not prevent or modify any stage in the development of endocarditis in the animals (Greenberg *et. al.*, 1987). In another study capsular polysaccharide induced immunity to *S. aureus* systemic infection was demonstrated (Lee *et. al.*, 1988). Immunization with capsular polysaccharide protected mice from non-lethal infection with the homologous strain.

Other later studies show that capsular polysaccharide may be involved specifically in the pathogenesis of infection of cardiac valves. Antibodies to *S. aureus* type 5 capsular polysaccharide were found to be protective in a modified rat endocarditis model (Lee *et. al.*, 1997). Rats given type-specific IgG showed a significantly lower prevalence of endocarditis than rats administered nonimmune IgG. Immunity was found to be type specific.

One can understand how antibodies to capsular polysaccharide are not protective if the model measures adherence to damaged tissue. On the other hand, it makes sense for antibodies specific for capsular antigens to protect in a model of systemic infection, due to opsonization of bacteria and subsequent clearance by the host immune system.

5.1.2.2 Collagen binding protein

The collagen-binding adhesin may play a minor role in infection of sites where the basement membranes are exposed as a result of tissue damage, such as occurs in traumatized heart valves. A Cna^- mutant attached to cardiac vegetations to the same extent as the wild type organism in a rat endocarditis model (Hienz *et. al.*, 1996). However, subsequent proliferation of the bacteria was reduced. Adhesion to collagen exposed in the basement membrane could be advantageous for the spread of the infection.

5.1.2.3 Involvement of regulatory genes

It has also been shown that mutants defective in either the *sar* locus (Cheung *et. al.*, 1994) or the *sar* and *agr* loci (Cheung *et. al.*, 1992), both of which regulate the expression of several virulence factors including coagulase, FnBPA and B and ClfA,

had a significant reduction in ability to cause experimental endocarditis in a rabbit infection model.

5.1.2.4 Role of fibronectin-binding proteins

The fibronectin-binding proteins are implicated in the pathogenesis of infectious endocarditis (Signas *et. al.,* 1989, Jonsson *et. al.,* 1991). The platelet-fibrin clot model described in this chapter has been used to investigate the role of the fibronectin receptor of *S. aureus* in binding to fibronectin-containing substrates (Rampyari *et. al.,* 1990). The potential role of fibronectin in the clot as a substrate for bacterial adherence was examined in an in vitro model system in which radiolabelled bacteria were seeded on a substrate composed of thrombin-induced clots of intact and fibronectin-depleted human plasma. The number of *S. aureus* Newman cells adhering to the clots was unaffected by the depletion of plasma fibronectin when the assay was carried out at low bacterial densities. However, bacterial attachment to plasma clots depleted of fibronectin was significantly less (up to 50%) than was attachment to clots made of intact plasma when the number of cells seeded on the substrate was increased. This indicated that fibronectin present in the plasma clots can in some instances serve as a substrate for bacterial adhesion. This observation is consistent with earlier studies (Toy *et. al.,* 1985) which showed decreased *S. aureus* adherence in a dose-dependent manner to plasma clots depleted of fibronectin.

The ligand binding site of FnBPA is composed of the D repeats near to the C-terminus of the protein. Each 38 amino acid D-repeat has some level of fibronectin-binding activity alone, but they are most effective in tandem as represented by the ZZ-FR fusion protein (Signas *et. al.,* 1989). The effects of various concentrations of the FnBPA peptides D1, D2, and D3 and the fusion protein ZZ-FR (composed of all D repeats) on the adherence of cells of strain Newman to plasma clots were studied (Rampyari *et. al.,* 1990). The results suggest that the fusion protein ZZ-FR could at the most reduce the number of bacteria adhering to the clots made from intact plasma by 50%. These results are consistent with the observed 50% maximal reduction in the number of bacteria adhering to fibronectin-depleted platelet-fibrin clots. The presence of the ZZ-FR fusion protein did not affect the number of bacterial cells adhering to clots made from fibronectin-depleted plasma. However, weak inhibition was seen when peptides corresponding to each of the fibronectin-binding D-repeats of the adhesin

were tested. None of the three synthetic peptides reduced the number of bacterial cells adhering to regular clots by more than 20%.

An in vivo study using a rat endocarditis model tested the ability of a fibronectin-binding defective mutant to cause infection (Kuypers and Proctor, 1989). A low fibronectin-binding mutant had a markedly reduced ability to adhere to traumatized rat heart valves, indicating that fibronectin binding is also important in vivo. Also, fibronectin-binding-deficient mutants of streptococci produced a lower rate of infection in a rat endocarditis model indicating the importance of fibronectin binding in the pathogenesis of this type of infection (Lowrance *et. al.,* 1990). However, in another study, it was shown that other binding functions of *S. aureus* may compensate for fibronectin binding in a catheter-induced infective endocarditis model (Flock *et. al.,* 1996). This was done by comparison of the adherence characteristics in vivo and virulence in a rat model of catheter-induced endocarditis of two isogenic strains of *S. aureus* differing in fibronectin binding. No differences were found between the two strains. The results strongly suggest that bacterial adherence to damaged heart valves may be multifactorial in nature.

Immunisation with fibronectin binding protein has been shown to protect against experimental *S. aureus* endocarditis in rats (Schennings *et. al.,* 1993). Antibodies were raised to the domains of FnBP responsible for ligand binding. These antibodies blocked *S. aureus* binding to immobilised fibronectin in vitro. Also, a significant difference in bacterial numbers within aortic valve vegetations between immunised and non-immunised groups was observed.

A model investigating the role of fibronectin in other foreign body infections has been performed. This study used a mutant of *S. aureus* defective in both *fnbA* and *fnbB*. This mutant demonstrated reduced adherence to ex vivo coverslips removed from subcutaneous chambers implanted in guinea pigs (Greene *et. al.,* 1995). This indicates that the FnBPs contribute to adherence to biomaterial that has been coated with host proteins in vivo. This has also been confirmed using ex vivo human catheters, for which degraded but functional fibronectin is the major determinant of staphylococcal adherence (Vaudaux *et. al.,* 1993).

5.1.2.5 Role of fibrinogen-binding proteins

When low numbers of bacteria are seeded on the plasma clot it appears that components other than fibronectin, such as fibrin, can efficiently mediate adherence of

bacterial cells. However, when these sites are saturated, it is then that incorporated fibronectin becomes an important alternate substrate for adherence of staphylococcal cells. The plasma clot adherence model described in this chapter has been also used in the study of fibrinogen binding by *S. aureus*. The pathogenic role of staphylococcal coagulase and ClfA has been assessed using adherence to solid-phase fibrinogen, a platelet-fibrin clot model and also a rat endocarditis model by P. Moreillon and co-workers (Moreillon *et. al.,* 1995). A coagulase-producing and ClfA-producing parent strain *S. aureus* Newman and a series of mutants defective in either coagulase, ClfA, or both were tested for their ability to attach in vitro to both purified fibrinogen and platelet-fibrin clots. It was found that the clumping factor-deficient mutants were significantly less adherent to solid-phase fibrinogen and also to plasma clots. Coagulase can bind to fibrinogen as well as to prothrombin, and is secreted into the extracellular medium (Boden and Flock, 1989, McDevitt *et. al.,* 1992). Coagulase-defective mutants were not altered in their in vitro adherence phenotype.

Clumping factor is also implicated in the pathogenesis of infective endocarditis. ClfA-defective mutants produced 50% less endocarditis than the parent organism when injected with an inoculum size infecting 80% of rats with the wild-type strain. Complementation of the ClfA defective mutant restored its adherence capability and in vivo infectivity to parent strain levels. A decrease in the infectivity of coagulase-deficient mutants of *S. aureus* has not been found (Moreillon *et. al.,* 1995, Vaudaux et. al., 1989) suggesting that coagulase is not involved in the pathogenesis of this type of infection.

The role of host matrix proteins in promoting staphylococcal adherence to biomaterials has been investigated using an ex vivo canine arteriovenous shunt model (Vaudaux *et. al.,* 1995). The results of this study suggested that fibrinogen is the foremost adhesion-promoting protein in short term blood-biomaterial interaction. It was previously thought that fibrinogen was not as important as fibronectin for promoting staphylococcal attachment. This was based on a study performed using ex vivo catheters removed from patients after a longer period of time in the body (Vaudaux *et. al.,* 1993). It seems that fibrinogen is more important in promoting the initial attachment of the organism and therefore could be considered crucial for the initiation of the infectious process.

Platelet aggregation by bacteria is also felt to play an important role in the pathogenesis of infective endocarditis. *S. aureus*-induced platelet aggregation requires

bacterial binding to fibrinogen, probably via the ClfA protein. However, this effect is not principally dependant upon the two major fibrinogen-binding domains on the platelet GPIIb/IIIa integrin receptor, the RGD and dodecapeptide recognition sites (Bayer *et. al.*, 1995).

5.1.3 Objective of this study

This section of the work set out to examine the role of ClfA in the adherence of *S. aureus* to a complex fibrinogen-containing substrate, such as platelet-fibrin clots. Initially, the data of P. Moreillon and co-workers with the ClfA$^-$ mutant was reproduced. Also, the effect of several inhibitors on bacterial adherence to the platelet-fibrin clots was studied. The inhibitors used were anti-Clf33(221-550) polyclonal IgG, mAbs 36 and 47 in combination, and also the purified Clf33(221-550) recombinant protein added extraneously to the platelet-fibrin clots. A mouse septicemia and renal infection model was also used in order to determine if passive administration of mAbs 36 and 47 could protect against *S. aureus* infection in vivo.

5.2 Results

5.2.1 Bacterial adherence to platelet-fibrin clots in vitro

A ClfA defective mutant of *S. aureus* strain Newman was found to have approximately a four-fold reduction in adherence to in vitro platelet-fibrin clots ($p<0.01$, Fig.5.1). This is similar to the result obtained by Philippe Moreillon and co-workers using the same strain, where a four- to six-fold difference was seen with the ClfA negative mutant. However, binding could not be reduced by adding purified recombinant Clf33(221-550) to the platelet-fibrin clots before addition of bacterial cells expressing ClfA (Fig. 5.2). No reduction in *S. aureus* binding was seen on incubation of platelet-fibrin clots with 1mg/ml Clf33(221-550) prior to addition of bacterial cells. No difference was seen compared to the negative control protein, 1mg/ml BSA.

When the bacterial cells were preincubated with anti-Clf33(221-550) polyclonal IgG, a 5 to 6-fold reduction in adherence occurred when a 1:100 dilution of IgG was preincubated with the bacterial cells ($p=0.026$, Fig. 5.3). No reduction in adherence was achieved when preimmune serum, a negative control, was used at a 1:100 dilution. Bacterial binding to the plasma clots could also be reduced using the combination of

Figure 5.1. Reduction in adherence to platelet-fibrin clots in vitro by a ClfA-defective mutant. Data shown represents the mean of 16 replicates and error bars show the standard deviation from the mean.

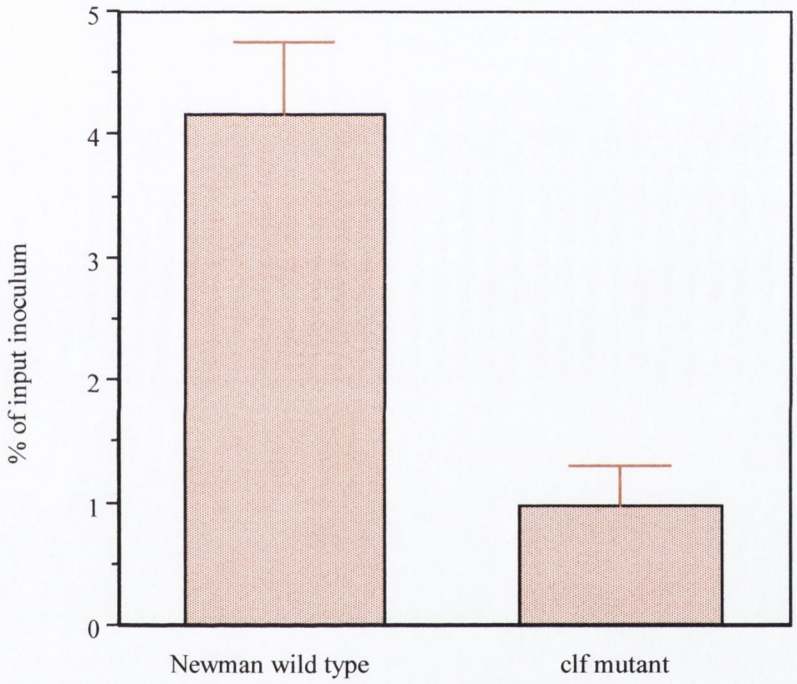

% of input inoculum
5
4
3
2
1
0
Newman wild type
clf mutant

Figure 5.2. Effect of the addition of 100μg/ml of purified Clf33(221-550) protein on bacterial adherence to platelet-fibrin clots in vitro. 100μg/ml of BSA is used as a control. Data shown is the mean of 14-17 replicates from a single experiment. Error bars are the standard deviation from the mean.

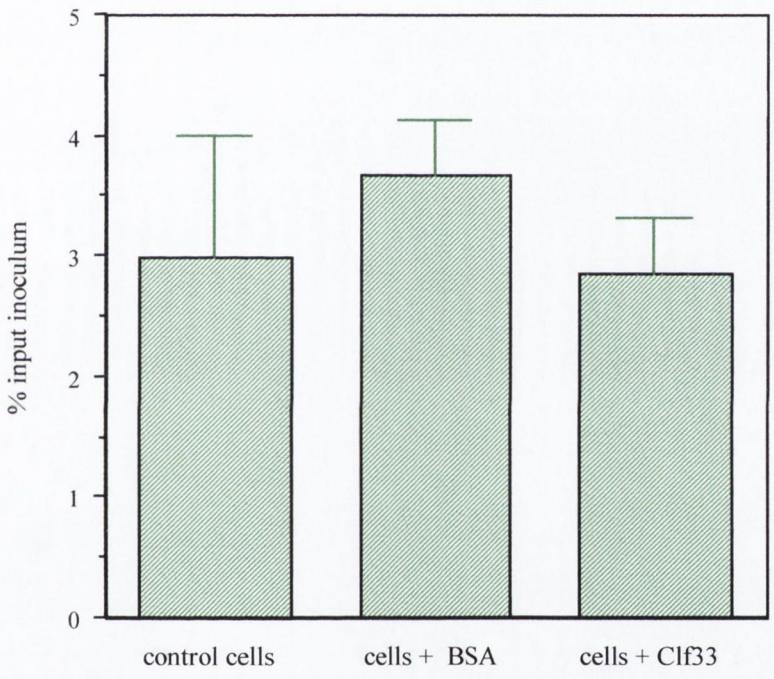
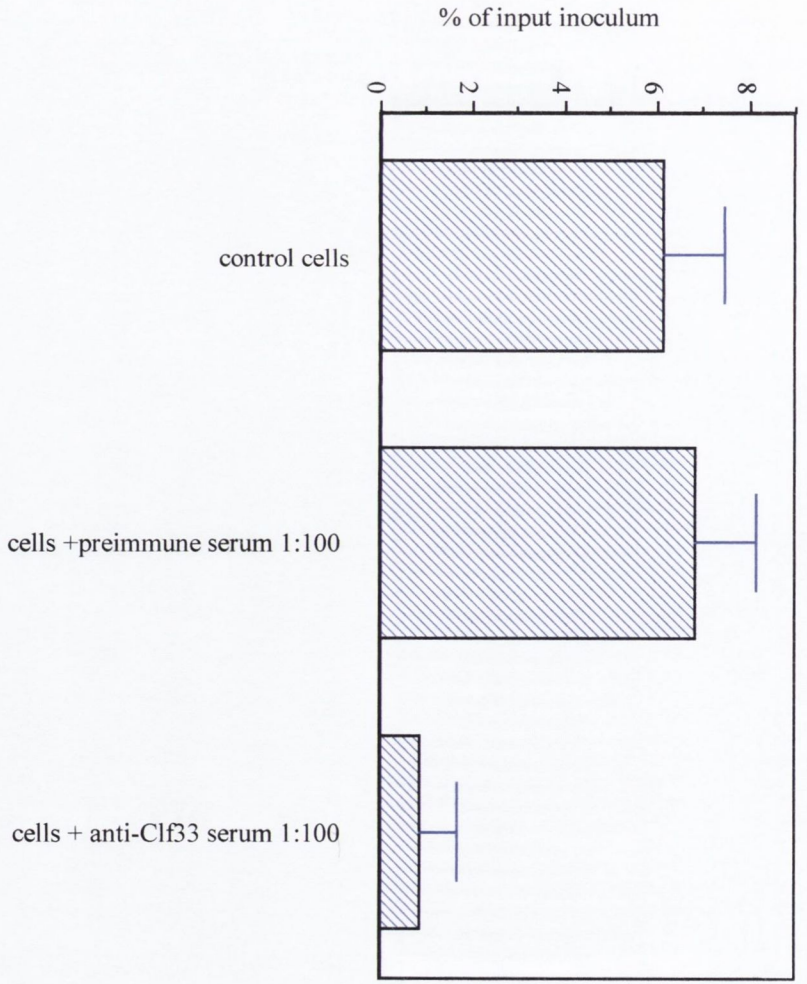

% input inoculum
5
4
3
2
1
0
control cells
cells + BSA
cells + Clf33

Figure 5.3. Inhibition of bacterial adherence to platelet-fibrin clots by anti-Clf33(221-550) polyclonal IgG. Data shown is the mean of 14-16 replicates. Error bars represent the standard deviation from the mean.


% of input inoculum
0
2
4
6
8
control cells
cells +preimmune serum 1:100
cells + anti-Clf33 serum 1:100

mAbs 36 and 47. At a final protein concentration of 20μg/ml, binding is 60% of control levels (p=0.002, Fig. 5.4).

5.2.2 Mouse renal infection and septicaemia model

A clinical *S. aureus* strain, F. Barnett, was used for this study. Cell culture supernatant containing mAbs 36 and 47 inhibited F. Barnett bacterial clumping in soluble fibrinogen at a 1:2048 dilution (Table 5.1). Neat cell culture supernatant from a hybridoma secreting a control IgG1 did not inhibit clumping. Adherence of this strain to fibrinogen was inhibited in vitro by mAbs 36 and 47 (Fig. 5.5). Serial twofold dilutions of the combined cell culture supernatants from these mAb hybridomas produced blocking in a dose-dependent manner.

The first pilot study was carried out in order to determine the minimum inoculum required in order to establish renal infection. Initially, three mice were injected by the intraperitoneal route with *S. aureus* at $6.0x10^6$, $6.0x10^7$ and $6.0x10^8$ c.f.u. in 0.5ml PBS. Bacteria could only be recovered from the kidneys of the animal that received the $6x10^8$ dose (Table 5.2). $4.5x10^4$ c.f.u. were found per gram of kidney 48h post-infection. Three mice were then given an inoculum of $1.4x10^9$ c.f.u./0.5ml. Similar kidney counts were obtained for each with an average of $9.3x10^4$. However, no correlation was seen between the level of bacteria in the kidney and visible signs of illness in the mice. Mouse 2A which was sacrificed 24h after inoculation due to its morbund state had less bacteria per gram of kidney than mouse 2C, which had the highest count and seemed well for the full 48h duration of the experiment.

The second pilot study was performed in order to determine the persistence of mAbs 36 and 47 in the bloodstream of the mice over the duration of the experiment. One mouse received 500μg/ml mAbs 36 and 47 subcutaneously, and levels of mAb in the bloodstream were monitored by ELISA over 120h (Fig. 5.6). Levels of mAbs seemed to persist well over the time period assayed. The amount of antibody in the bloodstream seemed to decrease approximately by half after each 24h time period.

Five mice were subsequently immunized with 500μg mAb36+500μg mAb47, and five control mice were immunized with 1mg of control IgG1. Forty-eight hours later, all ten mice were then challenged with $4x10^8$ c.f.u. in 0.5ml PBS by the intraperitoneal route. Mice were then sacrificed after 48h, kidneys homogenized, diluted and plated and qualitative blood cultures were also performed. Overall, no

Figure 5.4. Inhibition of bacterial adherence to platelet-fibrin clots in vitro by a final concentration of 20μg/ml anti-Clf33(221-550) mAbs 36 and 47 in combination. Data shown is the mean of 10-14 replicates. Error bars represent the standard deviation from the mean.

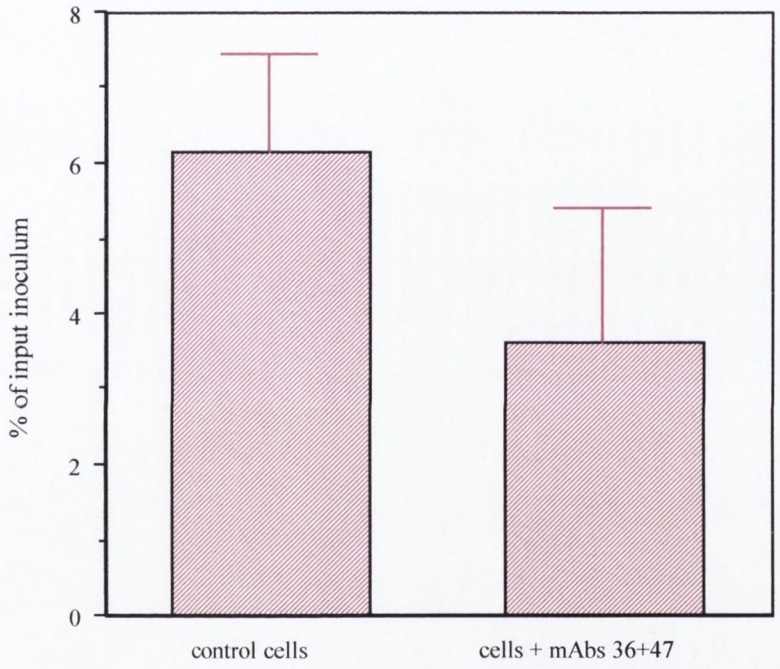

8
6
4
2
0
% of input inoculum
control cells
cells + mAbs 36+47

TABLE 5.1. Inhibition of *S. aureus* strain F. Barnett clumping in soluble fibrinogen.

supernatant	inhibiting dilution
36+47	1:2048
control IgG1	>neat

TABLE 5.2. Data from mouse infection model pilot studies to determine the minimum input inoculum to establish renal infection.

mouse	input inoculum (c.f.u./ml)	visible illness	c.f.u. recovered/g kidney
1A	$6.0x10^6$	no	-
1B	$6.0x10^7$	no	-
1C	$6.0x10^8$	no	$8.9x10^4$
2A	$1.4x10^9$	yes (24h)	$6.4x10^4$
2B	$1.4x10^9$	yes (48h)	$8.6x10^4$
2C	$1.4x10^9$	no	$1.3x10^5$

average pilot 2=$9.3x10^4$ +/- 3.4

Figure 5.5. Inhibition of *S. aureus* strain F. Barnett binding to fibrinogen by anti-Clf33(221-550) mAbs 36 and 47 in combination.

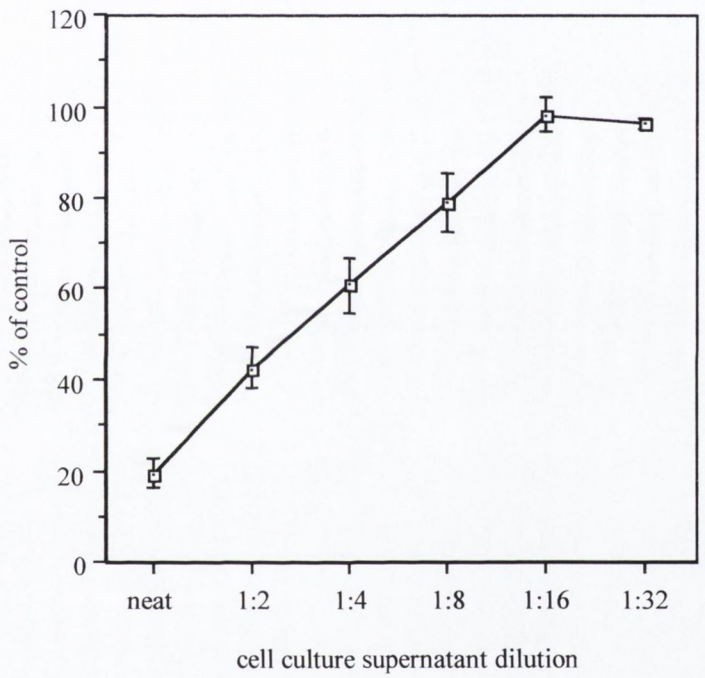
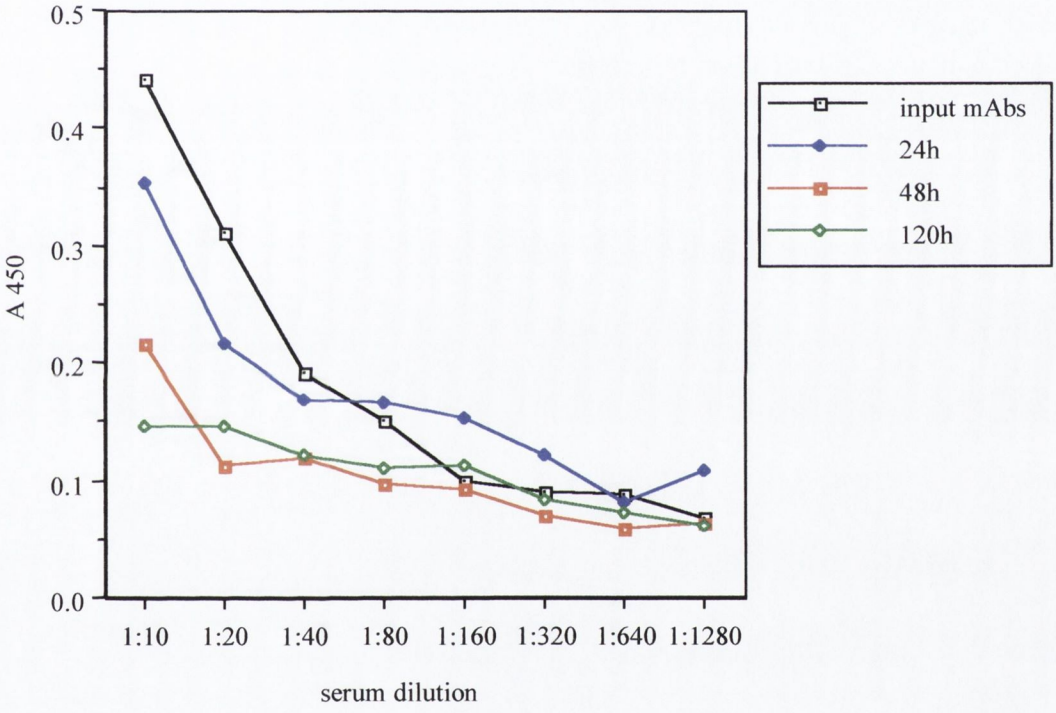

120
100
80
60
40
20
0
% of control
neat
1:2
1:4
1:8
1:16
1:32
cell culture supernatant dilution

Figure 5.6. Pharmokinetics of mAbs 36 and 47 persistence in the mouse bloodstream. The mouse received an intramuscular injection of 500μg/ml total IgG (250μg mAb36 +250μg mAb47 in 1ml PBS).


0.5
0.4
0.3
0.2
0.1
0.0
A 450
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
serum dilution
input mAbs
24h
48h
120h

significant difference was observed in counts between immunized and control groups of mice ($p=0.165$, Fig. 5.7). No correlation was observed between levels of mAb present in the immunized group and the number of bacteria isolated from the kidneys (Table 5.3). Also, the blocking mAbs in the bloodstream did not protect the vaccinated mice against bacteraemia. In fact, more of the vaccinated group of mice had positive blood cultures (4/5) than the control group (2/5).

5.3 Discussion

The result achieved in this study with the platelet-fibrin clot model using the *clfA* mutant was similar to that published by Philippe Moreillon and co-workers (Moreillon *et. al.,* 1995). In their study, 2.0% of the input inoculum of the wild-type organism expressing ClfA was found to be adherent to the rat platelet-fibrin clots. The *clfA* mutant had an adherence ratio of 0.6%. This is approximately a four-fold reduction that is statistically significant at the $p<0.05$ level. In this experiment, the wild type adhered to the canine plasma clots at 4.2% of the input inoculum and the *clfA* mutant at 0.96%, showing the same approximate four-fold reduction as was found for the rat plasma clots. This data is also statistically significant at the $p<0.05$ level.

Moreillon and co-workers used blood drawn from the same pool of donor rats for all of their experiments in order to standardise their plasma clots. Unfortunately, it was not practical to do the same in this study, as an animal with a larger blood volume was required. Canine blood was chosen as the plasma source as it was known from a previous study that the *S. aureus* Newman ClfA protein bound to fibrinogen from this species (Vaudaux *et. al.,* 1995). The different plasma source used could explain why the basal level of adherence in this study with the wild-type organism (4.2%) was higher than that found for rat plasma (2.0%). The amino acid sequence of the fibrinogen γ-chain shows strong homology between species, with 70% sequence identity in key regions of the γ-chain between lamprey and human (Strong *et. al.,* 1984). However, the differences present could explain altered binding characteristics. *S. aureus* Newman ClfA may be able to bind with slightly higher affinity to canine fibrinogen than to rat fibrinogen.

A modification of the method used previously was performed. Originally, Moreillon and co-workers described dehydration of the plasma clots at 37°C for 4h,

Figure 5.7. Bacterial counts in kidneys of mice passively immunized with mAbs 36 and 47 in combination or control IgG1. The data shown is the mean from each group of mice (n=5) and error bars show the standard deviation from the mean.

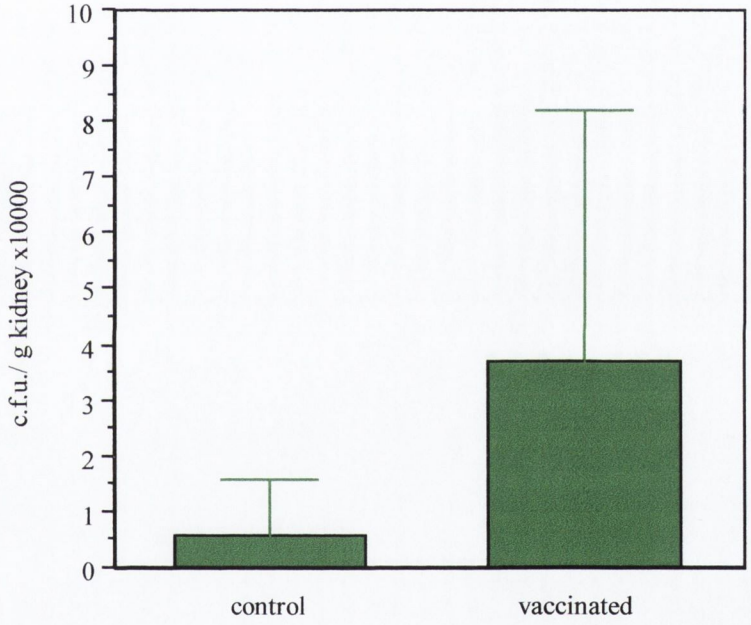

10
9
8
7
6
5
4
3
2
1
0
c.f.u./ g kidney x10000
control
vaccinated

TABLE 5.3. Data summary for individual mice from renal infection model.

mouse	bacteria recovered (c.f.u./g kidney)	serum ELISA (A 450)	blood culture
V1[a]	$6.9x10^4$	0.101	++
V2	$3.5x10^2$	0.106	-
V3	$9.0x10^3$	0.162	+(51)[b]
V4	$4.4x10^3$	0.234	+(31)
V5	$1.0x10^5$	0.119	+(12)
C6	$2.4x10^4$	0.007	+(72)
C7	$3.2x10^3$	0.003	-
C8	$9.9x10^1$	0.009	-
C9	$3.2x10^2$	0.013	++
C10	0	0.016	-

[a] V, vaccinated; C, control.

[b] Figure in brackets gives number of colonies formed from 10µl of blood. ++ shows bacterial growth that was too dense to count individual colonies.

with subsequent storage at 4°C until the adherence experiment was performed. Addition of bacteria suspended in buffer then presumably rehydrated the clot. In this study, it was found that the clots did not maintain their integrity when this method was used. Intact clots were required for the success of the experiment, so in subsequent experiments, no dehydration step was included. The platelet-fibrin clots were used for assay immediately after formation. This could also be a factor in the higher basal level of adherence in this assay as compared to Moreillon's data.

No inhibition of bacterial attachment to plasma clots was seen when the minimum fibrinogen-binding truncate, Clf33(221-550) was incubated with the clots prior to addition of bacteria expressing ClfA. When fibrinogen is converted to fibrin within the environment of a platelet-fibrin clot, the γ-chain C-terminus may not be accessible to the purified Clf33(221-550) truncate. However, native ClfA on *S. aureus* cells has the ability to bind to the clots. Also, as known from circular dichroism studies with ClfA truncated proteins, Clf33(221-550) has a less flexible conformation than full length region A (Nanavaty, Personal communication). This may explain why the native receptor on the bacterial cell surface can bind to the fibrinogen/fibrin present on the surface of the platelet-fibrin clot, due to its greater ability to fold as required. Bacterial binding to fibronectin in platelet-fibrin clots could be reduced by incorporating a fusion protein corresponding to the D-repeats, containing the fibronectin-binding site, into the plasma before formation of the clot (Rampyari *et. al.,* 1990). This approach would perhaps have yielded more success that trying to add Clf33(221-550) after polymerisation of the clot.

Another possibility may be that the C-terminal γ-chain of fibrinogen is not accessible to Clf33(221-550) when fibrinogen monomers assemble to become fibrin. Clotting occurs in two distinct stages (Voet and Voet, 1990). A "soft" clot, as its name implies, is rather fragile. It is rapidly converted to a more stable "hard" clot by the covalent cross-linking of neighbouring fibrin molecules in a reaction catalysed by fibrin-stabilising factor (FSF of factor $XIII_a$). This transamidase initially joins C-terminal segments of adjacent γ-chains by forming isopeptide bonds between side chains of a Gln residue on one γ-chain and a Lys residue on another. Two such symetrically equivalent bonds are rapidly formed between each neighbouring pair of γ-chains. The α-chains are also cross-linked to one another but at a much slower rate. (Kloczewiak

et. al., 1987). Fibrin cross-linking is physiologically important. Individuals deficient in FSF have a pronounced tendency to bleed.

The adhesion of *Plasmodium falciparum*-infected erythrocytes to ICAM-1 is thought to be important in the pathogenesis of this disease (Craig et. al., 1997). It was found that recombinant soluble ICAM-1 could not block adhesion of infected erythrocytes to solid-phase ICAM-1 in vitro. It has been proposed that this is due to ICAM-1 dimers in solution having rapid k_{off} binding rates such that these soluble molecules are unable to compete with the multivalent nature of ICAM-1 bound to a plastic surface. Clf33(221-550) bound to fibrinogen on a platelet-fibrin clot may not be able to compete effectively with native ClfA on the bacterial cell surface.

In Fig 5.3, a marked increase in the basal level of adherence for the control cells of 6.13% of the input inoculum is seen as compared with Figs 5.1 (4.16%) and 5.2 (3.67%). The difference probably reflects plasma difference between dogs, as it was not practical to use plasma from the same animal twice. Pooling of plasma from several different animals each time the clots were made, as done by Moreillon and co-workers with the rat plasma used for their platelet-fibrin clots, would probably have yielded more consistent basal levels of bacterial adherence to the clots. Anti-Clf33(221-550) polyclonal IgG at a 1:100 final dilution produced approximately a six-fold reduction in adherence to the platelet-fibrin clots (Fig. 5.1). This is a slightly larger reduction than that achieved by mutating ClfA. It is a possibility that polyclonal IgG raised against residues 221-550 could cross-react with the fibronectin-binding proteins. 31% sequence identity occurs between region A of FnBPA and residues V249-N349 of ClfA (McDevitt *et. al.,* 1994). Anti-Clf33(221-550) polyclonal IgG could conceivably block FnBP binding to plasma clots. However this effect, if any, would need to be steric in nature as the fibronectin-binding site of this protein is located in the D-repeats towards the C-terminus, and not the A region where the homology between the two proteins is greatest (Signas *et. al.,* 1989).

Residual adherence seen both with the ClfA$^-$ mutant and blocking attachment with anti-Clf33(221-550) polyclonal IgG could be attributed to other surface receptors recognising components of the platelet-fibrin clot. The fibronectin-binding proteins seem to be the main candidate for mediating this secondary adherence. Rampyari and co-workers performed their platelet-fibrin clot adherence assay in microtiter wells using radiolabelled bacteria. $2x10^7$ cells/50µl PBS were added to clots made in 5mm

diameter microtiter wells. At low bacterial densities it was found that other components of the clot, such as fibrinogen/fibrin, were mediating adherence more efficiently than fibronectin. In this study, $5x10^3$ cells/2ml PBS were added to clots polymerised in 30mm plastic dishes. This is comparitively low density seeding as compared to the fibronectin platelet-fibrin clot study.

Other components present in the matrix of a platelet-fibrin clot could also mediate adherence of *S. aureus* and other organisms expressing similar binding proteins on their cell surfaces. For example, *S. aureus* can bind to thrombospondin, the major platelet protein released from activated platelets (Herrmann *et. al.,* 1991). Platelets bound to plastic surfaces promoted attachment of *S. aureus* cells (Herrmann *et. al.,* 1992). This interaction was enhanced by plasma, especially by fibrinogen/fibrin, perhaps acting as a bridge between the immobilized platelet and the bacterial cell. Another *S. aureus* protein which may be involved is MAP, a broad-specificity protein which has been shown to bind fibrinogen, fibronectin, thrombospondin, vitronectin and collagen in Western ligand blotting experiments (Homonylo McGavin *et. al.,* 1993). However, there is no evidence to suggest that this protein is surface-located.

The combination of mAbs 36 and 47 at a final concentration of 20μg/ml produced a 40% reduction in bacterial adherence. This result is in agreement with the level of inhibition seen in the soluble fibrinogen clumping assay and the solid-phase attachment assay using this combination of mAbs (see sections 4.2.1 and 4.2.2). This result, although showing large variation, is statistically significant. Due to the inherent variation in this platelet-fibrin clot attachment assay, a large sample number must be used. Usually, between 12 and 16 plates are used for any one treatment to achieve statistically relevant data.

An in vivo experiment, a mouse bacteraemia and renal infection model, was also conducted. Unfortunately, when the infection was performed on a control and vaccinated group of animals, the reproducible infectivity data achieved in the pilot studies could not be repeated. The levels of mAbs 36 and 47 present in the bloodstream did not appear to have any influence on the number of bacteria recovered from the kidneys of the animals or on the presence of bacteria in the bloodstream of the animals. As a result, this group of experiments was discontinued. ClfA may not be a relevant virulence factor in this type of intraperitoneal infection model. Previously, a murine model of hematogenously induced *S. aureus* sepsis and arthritis was succesfully

used to show that neutrophils have a crucial protective role in the early phase of *S. aureus* infection (Verdrengh *et. al.,* 1997). Similar animal models of septicaemia have also been used to demonstrate the efficacy of passive and active immunization against type 5 capsular polysaccharide (Fattom *et. al.,* 1996). Also, virulence genes involved in a murine model of *S. aureus* bacteraemia were identified using signature-tagged mutagenesis (Mei *et. al.,* 1997). Unfortunately, none of the known staphylococcal adhesins were shown be involved. Perhaps this type of animal model is not relevant to the study of adherence by *S. aureus*. However, it has been demonstrated that ClfA has a role in the pathogenesis of infective endocarditis (Moreillon *et. al.,* 1995). Unfortunately, it was not feasible to perform an endocarditis model and no in vivo protection data using the mAbs has been generated as a part of this particular study. This technique is extremely specialized and could not be performed as part of this work. Perhaps the rat endocarditis model would have been more relevant in order to evaluate anti-ClfA antibody therapeutic potential in vivo.

Recently, preliminary data has shown that active immunization with Clf40(40-559) is protective against intravenous challenge with *S. aureus* strain MRSA 601 (Patti, personal communication). Only 1/9 of the immunized mice died compared with 5/10 of the control unvaccinated animals. Also, control mice had significantly more body weight loss and higher bacterial load in the kidneys than the vaccinated group. Using the same model, passive immunization with 10ng IgG raised against Clf30(332-550) also protected against *S. aureus* infection. Out of the control group of mice 6/11 died while only 1/11 of the vaccinated mice did not survive bacterial challenge. This model offers greater control over the number of bacteria in the bloodstream. The peritoneal infection is artificial, as this never occurs in human staphylococcal infections.

CHAPTER 6

IDENTIFICATION OF CLUMPING FACTOR A EPITOPES RECOGNISED BY ANTI-CLUMPING FACTOR A MONOCLONAL ANTIBODIES

6.1 Introduction

Antibodies have long been recognised as sensitive probes of the structure of complex molecules. Three pioneering studies in the field of protein structure and folding were as follows; observations that antibodies against apomyoglobin induced the release of heme from myoglobin, investigations on the kinetics of folding of serum albumin and the demonstration that peptide fragments isolated from staphylococcal nuclease can adopt a native-like immunoreactive conformation (Goldberg, 1991). However, these studies were hampered by the heterogenous nature of the polyclonal immune serum used. The advent of hybridoma technology made it possible to obtain a panel of monospecific antibodies each directed against a given epitope.

Monoclonal antibodies with thoroughly characterized target specificities can be used as powerful probes of protein structure and conformation, and the changes that occur on ligand-binding. Using mAbs it has been shown that when thrombin cleaves antithrombin, the latter undergoes a conformational change that affects the heparin-binding site (Peterson and Blackburn, 1987). mAbs have also been employed to detect subtle conformational changes upon zinc binding to carboxypeptidase A (Solomon *et. al.,* 1988) and calcium binding in the calmodulin-dependent protein kinase II (Le Vine *et. al.,* 1988). An extensive comparison of the conformation and function of soluble and membrane-bound F1-ATPase has been made (Godinot *et. al.,* 1986). Also, mAbs have been used to demonstrate long-range perturbations of the conformation of chemically modified cytochrome c (Collawn *et. al.,* 1988) and variations in the conformation of the hypervariable regions of immunoglobulins (Cazauboun., 1988). The following sections discuss various methods that can be used in order to locate epitopes on protein antigens recognised by mAbs that interfere with protein function.

6.1.1 Localisation of mAb epitopes using recombinant deletion fragments.

A very useful way to crudely locate a protein domain containing a specific mAb epitope is by investigating binding of mAbs to proteolytic fragments of an antigen or indeed recombinant fragments generated by deletion mutagenesis. This is especially useful in identifying the location of a mAb that interferes with protein activity. For example, using mAbs as a tool for the functional analysis of pneumolysin, three regions of the toxin sequence involved in the lytic mechanism were identified (De Los Toyos *et. al.,* 1996). These three mAbs interfered with the activity of the toxin, and therefore recognised epitopes involved in the function of the protein. Two of the mAbs were

mapped to the cell-binding site of the toxin, while one was involved in binding to the lytic site of the protein. This information was generated by Western immunoblotting using mAb reactivity against recombinant fragments of pneumolysin and also proteolytically nicked toxin.

Neutralization epitopes of the VP2 protein from infectious pancreatic necrosis viruses have also been characterised using *E.coli* expressed deletion fragments of VP2 and monoclonal antibodies (Frost *et. al.,* 1995). The results from this study demonstrated that the central third of VP2 contained several partly overlapping neutralization epitopes, both variable and conserved. Specific monoclonal antibodies to the major outer membrane porin protein of *Pseudomonas aeruginosa*, OprF, were localised by immunodetection of OprF fragments generated by papain and cyanogen bromide cleavage (Rawling *et. al.,* 1995). Seven mAbs recognising conformational epitopes were localised to regions of OprF 42-90 amino acids in length, located on two adjacent loops as seen from a structural model of this protein. Recombinant fusion proteins of high molecular weight adhesion proteins (HMW) of nontypeable *Haemophilis influenzae* were also used to map mAbs reacting with surface-exposed epitopes of these molecules (Barenkamp and St Geme, 1996). One mAb, designated AD6, reacted with the last 75 amino acids of the C-terminus of both HMW1 and HMW2. Another mAb, 10C5, mapped to a 155-amino acid segment of HMW1 only. This 10C5 epitope was also present on 40% of strains reactive with AD6. Identification of shared surface-exposed epitopes shared among strains of pathogenic bacteria may be important for the development of recombinant or synthetic peptide-based vaccines against disease caused by the majority of strains.

A monoclonal antibody which actually enhanced binding of fibronectin to the FnbA protein of *Streptococcus dysgalactiae* was mapped to an area just N-terminal to the primary ligand-binding site (Speziale *et. al.,* 1996). Binding of this important mAb to a set of recombinant truncated proteins with C-terminal serial deletions was examined. The epitope was found within a 81 amino acid long segment adjacent to the repeats containing the fibronectin-binding site.

6.1.2 Precise mapping of linear epitopes using overlapping synthetic peptides.

Monoclonal antibodies that recognise linear sequences can be mapped to their exact corresponding epitopes within the protein by the use of overlapping synthetic

peptides. Unfortunately, this is not a good method for the localisation of antibodies recognising conformational or non-contiguous antibody binding sites. Epitopes involved in the pore formation of listeriolysin secreted by *Listeria monocytogenes* were mapped using neutralizing monoclonal antibodies and 166 overlapping synthetic dodecapeptides (Darji *et. al.,* 1996). A region was defined using this method that had not been previously implicated in mediating haemolytic activity. This peptide scanning can be useful in identifying epitopes important in the infection process, by binding polyclonal serum or convalescent patient serum to the peptide series. Immunologically relevant human B-cell determinants on the S1 subunit of pertussis toxin were identified using overlapping synthetic decapeptides (Raupach and Schmidt, 1994). Human anti-pertussis sera predominantly recognized linear immunodominant epitopes located in three separate segments spanning amino acids 3-16, 21-30 and 211-222. The third epitope was indicative of acute infection only, and may prove a useful method for the diagnosis of acute *Bordetella pertussis* infection.

6.1.3 Studies of mAb binding to site-directed mutant proteins

Altered mAb binding to site-directed mutants of a protein can yield important insight into protein function and conformational changes caused by the mutation of key residues involved in structure and activity. TSST-1, a toxin secreted by *S. aureus*, is a bifunctional molecule. This protein binds to the T-cell receptor on the surface of leucocytes and also to HLA-DR on antigen presenting cells. Both activities could be correlated directly with a panel of mAbs that discriminated the superantigen functional sites on TSST-1 specific for either HLA-DR or T-cell receptor interaction sites. These mAbs were localised to these domains by virtue of their reactivity with a panel of site-specific mutant TSST-1 molecules (Shimonkevitz *et. al.,* 1996). The epitopes recognised by four out of the five mAbs characterized in this study could be defined by the mutations.

6.1.4 Use of mAbs and protein complexes in epitope mapping

When a protein binds to its corresponding ligand, this may induce a conformational change in the molecule. This may expose new epitopes or conceal epitopes present on the non-complexed form of the molecule. The domain organization of a 900kDa complex, botulinum neurotoxin serotype A was studied by using a panel of monoclonal single-chain variable fragments (Chen *et. al.,* 1997). These were reacted

with various combinations of the domains of the protein recombined in vitro. Only 20 of the antibody fragments reacted with the toxin in complex with the non-toxic portion. All of the covered epitopes were located to the binding domain of botulinum neurotoxin, suggesting that the binding domain is in direct contact with the non-toxic portion of the complex. A ligand-induced conformational change in the ferrichrome-iron receptor, FhuA, of *E. coli* K-12 was also detected by a similar approach (Moeck *et. al.,* 1996). The addition of ferrichrome to purified FhuA reduced the ability of certain anti-FhuA monoclonal antibodies to bind to the receptor.

6.1.5 Motifs involved in the ClfA-fibrinogen interaction.

Three motifs have been found within 221-550 of ClfA that may have a role in fibrinogen binding. Both ClfA and the leucocyte integrin $\alpha_{IIb}\beta_3$ interact with the C-terminal portion of the fibrinogen γ-chain (D'Souza *et. al.,* 1990, McDevitt *et. al.,* 1997). Both proteins contain a motif known as an EF hand, which is found in eukaryotic Ca^{2+} binding proteins. This motif contains 13 amino acids, where positions 1, 3, 5, 7 and 12 are occupied by oxygenated residues. A solvent molecule is associated with residue 9 by a hydrogen-bond (Barioch, 1991). These residues form a sphere of co-ordination for Ca^{2+} and are flanked by α-helices. Within this sequence two types of Ca^{2+} binding sites are found. Structural sites bind Ca^{2+} with high affinity while regulatory sites bind with low affinity. ClfA contains several putative EF hand loops, of which one located at residues 310-321 has been studied (O'Connell *et. al.,* 1998) Mutagenesis of Ca^{2+} co-ordinating residues within this motif result in reduced fibrinogen binding by ClfA and reduced Ca^{2+} regulation. The end of this motif overlaps the highly conserved TYTFTDYYD motif found in all proteins belonging to the SDR multigene family (Ni Eidhin *et. al.,* 1998, Josefsson *et. al.,* 1998). Deletion mutants and site-directed mutants of the EF hand motif in recombinant truncated ClfA proteins 40-559 and 221-559 caused a significant reduction in affinity of these proteins for whole fibrinogen and the fibrinogen γ-chain peptide. It has been shown that Ca^{2+} causes a conformational change in ClfA when bound to a site in or near the EF hand motif. However, this Ca^{2+} inhibitory effect is seen with the 40-559 truncate, not in 220-559, implying that the latter has a different conformation. ClfA is then in a conformational state incapable of interacting with the C-terminal γ-chain of fibrinogen, as the γ-chain peptide causes a conformational change in ClfA.

ClfA posesses a second motif which is implicated in its interaction with fibrinogen (Altieri *et. al.,* 1993). Ligand binding by the leucocyte integrin $\alpha_M\beta_2$ involves a 200-residue insertion of an A (or I) domain in the α chain. This domain contains a novel cation-binding metal ion-dependent adhesion site (MIDAS) motif (Lee *et. al.,* 1995). The integrity of the MIDAS motif is essential for integrin-ligand binding. ClfA has a MIDAS-like consensus sequence that may be involved in fibrinogen binding. Site-directed mutagenesis of amino acids within this motif resulted in a significant reduction in fibrinogen binding by the recombinant ClfA proteins (O'Connell, Personal communication). Interestingly, no defect in γ-chain peptide binding or Ca^{2+} inhibition of the fibrinogen-ClfA interaction occurred on mutation of residues of this motif.

The C-terminus of region A seems to be somehow involved in fibrinogen binding. C-terminal recombinant truncates containing residues 221-484 and 221-525 can no longer interact with fibrinogen (O'Connell, personal communication, Hartford, personal communication). Also, a recombinant protein consisting of ClfA 380-550 can absorb neutralizing polyclonal antibodies but cannot itself bind to fibrinogen. Another set of residues located at residues 526-532 in the C-terminus of ClfA region A have been shown to have a role in the ClfA-fibrinogen interaction. When E526 and V527 of this EVA motif is mutated in ClfA, there is a marked reduction in the binding ability of the purified recombinant proteins to whole fibrinogen and also the γ-chain alone. However, Ca^{2+} regulation of the ClfA-fibrinogen interaction is retained and no conformational change occurs. This was also shown when the mutations were transferred into a ClfA molecule expressed on the bacterial cell surface (Hartford, Personal communication).

6.1.6 Objective of this study

This section describes the attempted localization of epitopes recognised by mAb6 raised against Clf42(332-559) and mAbs 36 and 47 raised against Clf33(221-550). This was initially achieved by the study of mAb binding to recombinant truncated ClfA proteins on Western immunoblots. Anti-Clf33(221-550) polyclonal IgG and mAbs were reacted with ClfA 25-mer overlapping peptides corresponding to residues 333-557 in a solid-phase assay. Inhibition of mAbs 36 and 47 binding to Clf41(221-559) by an EF hand peptide was investigated. Subsequently, mAb binding to ClfA site-directed mutant proteins was studied. Also Clf41(221-559) complexed with whole

fibrinogen or the C-terminal γ-chain fibrinogen peptide (396-412) were used to study mAb-ClfA interactions.

6.2 Results

6.2.1 Antigenicity profile of residues 221-559

The algorithm of Jameson and Wolf predicts likely surface-exposed antigenic epitopes of a protein (Jameson and Wolf, 1988). There are four main regions within residues 221-424 with an antigenicity index of >1.300 as predicted by this model (Fig. 6.1), one of which is recognised by anti-Clf33(221-550) blocking polyclonal IgG (Fig. 6.3). The residues of the EF hand and the overlapping TYTFTDYYD motif are also predicted to be surface exposed. There are seven possible immunogenic regions between residues 332-559, of which two linear epitopes are recognised by polyclonal IgG (Fig. 6.3). These estimations assume that an epitope constitutes at least 5 consecutive amino acid residues with a positive antigenicity index (Voet and Voet, 1990, Frost *et. al.*, 1995) with a value of >1.300.

6.2.2 Epitope localisation by binding of mAbs to truncated ClfA proteins on Western Immunoblots

Various ClfA truncates (Table 2.2) were used in order to begin defining the epitopes recognised by mAbs6, 36 and 47. The epitope recognised by mAb6 could be localised to residues 332-525 by this method (Table 6.1). mAbs 36 and 47 bound to the same set of truncates. By virtue of binding to Clf28 (23-424), both of these mAbs could be localised to a site between residues 221-424. Anti-Clf33(221-550) polyclonal IgG bound to all recombinant proteins.

6.2.3 Binding of anti-Clf33(221-550) polyclonal IgG and mAbs to immobilized ClfA peptides

This experiment was performed in order to determine if the anti-Clf33(221-550) polyclonal IgG or the blocking mAbs recognised linear epitopes present in amino acids 333-557 of ClfA region A. An ELISA was performed to investigate if antibody bound to this series of synthetic 25-mer peptides spanning residues 333-557 (Table 2.3) or to a peptide corresponding to the EF hand residues 310-321 that were immobilised on a

Figure 6.1. Predicted antigenic epitopes within ClfA residues 221-559. Domains defined by polyclonal antibodies (pAb) and mAbs 6, 36, and 47 are shown. Motifs of significance are also illustrated.

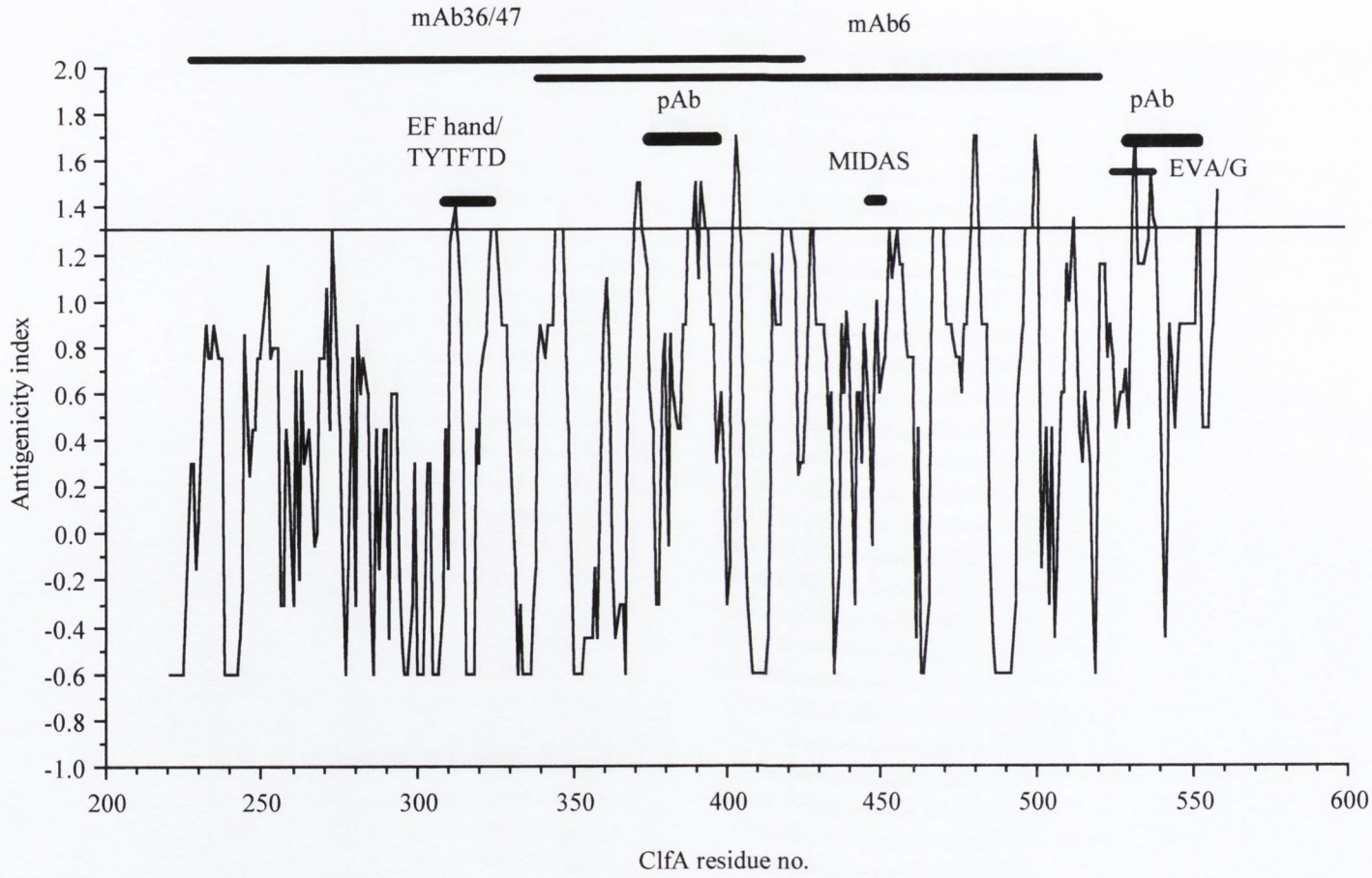

mAb36/47
mAb6
pAb
pAb
EF hand/
TYTFTD
MIDAS
EVA/G
Antigenicity index
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
200
250
300
350
400
450
500
550
600
ClfA residue no.

TABLE 6.1. Reactivity of blocking mAbs with truncated ClfA proteins.

	mAb6	mAb36	mAb47
Clf27 (420-550)[a]	-	-	-
Clf28 (23-424)	-	+	+
Clf29 (23-308)	-	-	-
Clf33 (221-550)[b]	+	+	+
Clf40 (40-559)	+	+	+
Clf41 (221-559)	+	+	+
Clf42.(332-559)	+	-	-
Clf43 (221-484)	-	+	+
Clf44 (272-559)	+	-	-
Clf63 (272-425)	-	ND	ND
Clf64 (272-484)	-	ND	ND
Clf65(420-559)	-	ND	ND
Clf(221-525)	+	+	+
mAb epitope within residues	332-525	221-424	221-424

[a] Numbers in brackets are ClfA residues present in recombinant molecule. Anti-Clf33(221-550) polyclonal IgG binds to all truncates.

[b] Constructs in bold type show fibrinogen-binding truncates.

polystyrene surface. Anti-Clf33(221-550) polyclonal IgG bound to 2 peptides within the series (Fig. 6.2). These correspond to residues 373-397 and 533-557. None of the mAbs bound to immobilized peptides by ELISA or dot-blot.

6.2.4 Effect of a soluble EF hand(310-321) peptide on mAb 36 and 47 binding to Clf41(221-559)

In order to determine if mAbs 36 and 47 were binding directly to the EF hand, these mAbs were preincubated with a soluble peptide corresponding to residues 310-321 of ClfA. A decrease in Clf41(221-559) binding of 29% ($p=0.05$) and 14% ($p=0.025$) was seen for mAbs 36 and 47 respectively (Fig. 6.3). No inhibition of either mAb was observed with a peptide corresponding to residues 373-397 of ClfA. This indicates that the epitopes recognised by both of these mAbs may overlap the EF hand.

6.2.5 Binding of polyclonal IgG and mAbs to immobilized ClfA site-directed mutant proteins

This study was performed in order to investigate whether mutations in functionally important sites in ClfA had an effect on binding of the blocking mAbs 6, 36 and 47 to ClfA. The Clf68 recombinant protein contained a quadruple site-directed mutation in the co-ordinating residues of the EF hand motif (D310A/D312A/T318A/D321A). Clf92 contained a double mutation in the putative MIDAS motif (D443A/S445A). The SM4 protein was also a quadruple mutant, with four residues in the extreme C-terminus of region A altered (E526L/V527S/A528R/G532Z).

Polyclonal IgG and mAbs were tested for their binding to these ClfA site-directed mutant proteins by ELISA. Polyclonal IgG, used as a control to show that protein had coated the ELISA plate wells, bound to all proteins (Fig. 6.4). The only major difference in binding of anti-Clf33(221-550) polyclonal IgG was to SM4 (approximately two-fold less). Differences seen probably reflect slight variations in protein concentration used for coating and/or different binding properties of the mutant proteins to the polystyrene microtiter plates caused by the amino acid changes.

The most dramatic differences seen were with the mAbs (Fig. 6.5). Again, mAbs 36 and 47 showed similar patterns. The binding of both of these mAbs was affected by a quadruple mutation in the EF hand. These mAbs did not bind to a recombinant protein, Clf51(Δ310-321), with a deletion in the EF hand loop residues on

Figure 6.2. Reactivity of anti-Clf33(221-550) polyclonal IgG with immobilized ClfA peptides. Data points represent the mean of triplicate ELISA wells. Error bars show the standard deviation from the mean.

A 450
0.0
0.2
0.4
0.6
0.8
1.0
1.2
protein/peptide
Clf33(221-550)
Clf42(332-559)
333-357
353-377
373-397
393-417
413-437
433-457
453-477
473-497
493-517
513-537
533-557
310-322

Figure 6.3. Inhibition of anti-Clf33(221-550) mAbs 36 and 47 binding to Clf41(221-559) by a soluble peptide corresponding to the EF hand (residues 310-321). A control peptide corresponding to ClfA residues 373-397 is used. 100μg/ml of each peptide is used. Data points are the mean of triplicate wells and are from one representative experiment. Error bars show the standard deviation from the mean.

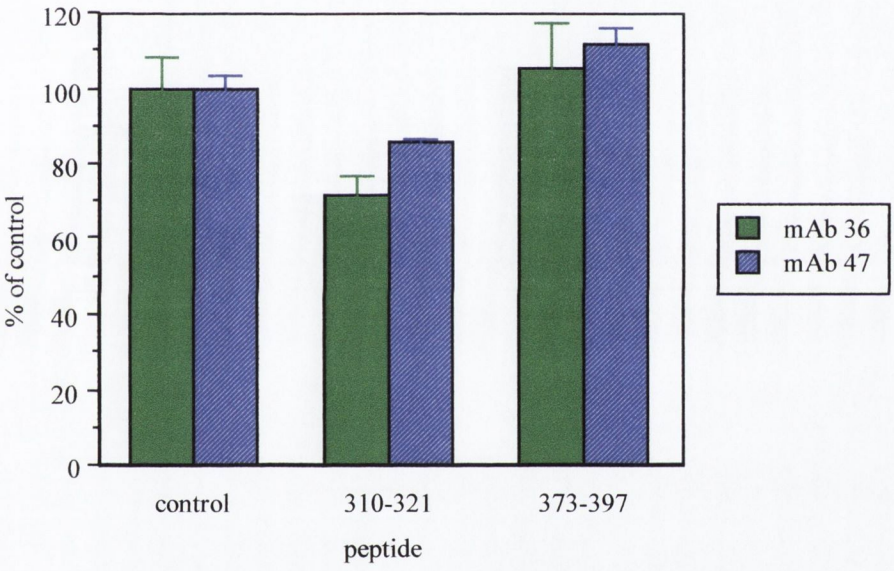

120
100
80
60
40
20
0
% of control
control
310-321
373-397
peptide
mAb 36
mAb 47

Figure 6.4. Binding of anti-Clf33(221-550) polyclonal IgG to ClfA mutant proteins. ELISA wells were coated with 10μg/ml of each protein. Data points are the mean of triplicate determinations from one ELISA and the error bars show the standard deviation from the mean.

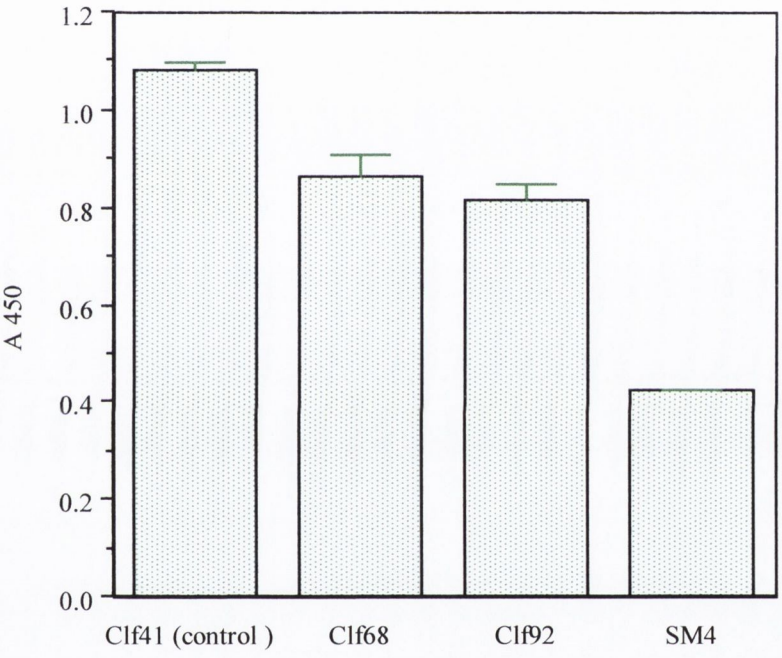
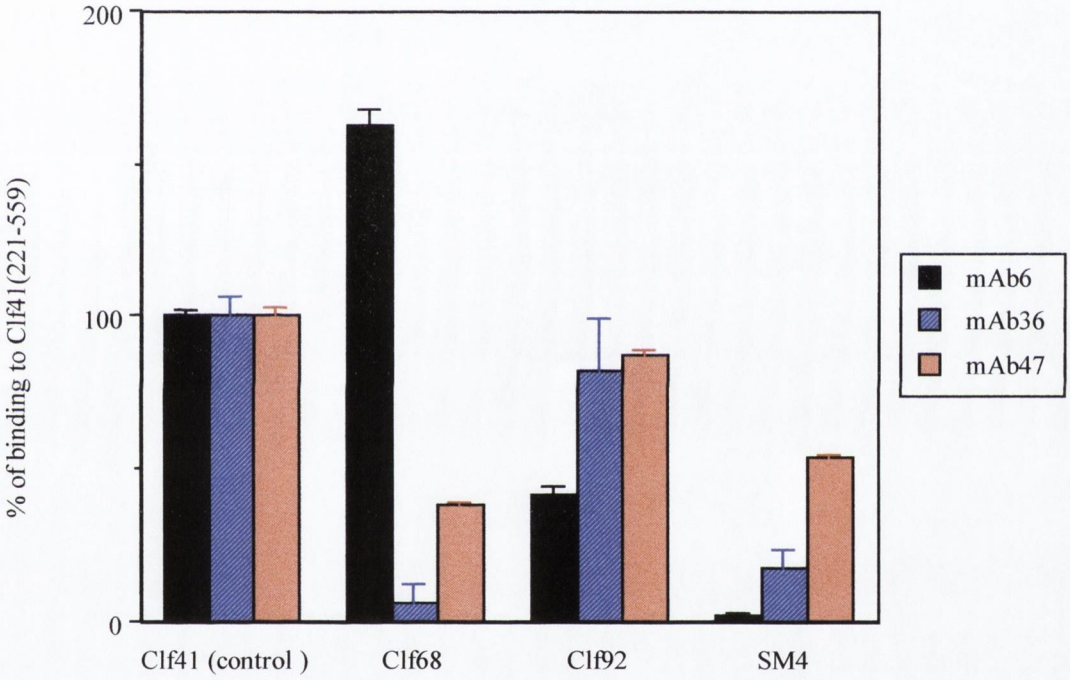

1.2
1.0
0.8
0.6
0.4
0.2
0.0
A 450
Clf41 (control)
Clf68
Clf92
SM4

Figure 6.5. binding of anti-ClfA mAbs 6, 36 and 47 to ClfA mutant proteins. Values shown are the mean of triplicate ELISA well readings. Error bars represent the standard deviation from the mean.


200
100
0
% of binding to Clf41(221-559)
Clf41 (control)
Clf68
Clf92
SM4
mAb6
mAb36
mAb47

Western immunoblots (Table 6.1). However, mAb36 also showed a decrease in binding to the SM4 mutant protein. This is not seen in the pattern shown for mAb47.

A completely different pattern was shown by mAb6. There was no reduction in mAb6 binding to Clf68. In fact, an increase in binding was seen. This mAb was also capable of binding to Clf51(Δ310-321) on Western immunoblots (Table 6.1). However, mAb6 binding was reduced more than half by the double mutation in the MIDAS motif, and almost completely abolished in the C-terminal SM4 mutant. In fact, mAb6 was extremely sensitive to changes in the C-terminal of region A. This is also reflected in the truncated protein Western immunoblotting data.

6.2.6 Binding studies of mAbs to Clf41(221-559) in complex with fibrinogen γ-chain peptide 396-411 or whole fibrinogen

A peptide corresponding to residues 396-411, the C-terminal 17 amino acids of the γ-chain of fibrinogen and whole fibrinogen were bound to Clf41(221-559) in solution. The complexes were then coated onto ELISA wells, and mAbs were bound. Anti-Clf33(221-550) polyclonal IgG was included as a control to indicate that differences observed were not due to differential coating of the complexes from Clf41(221-559). Polyclonal IgG showed a similar level of binding to each complex as compared to uncomplexed Clf41(221-559) (Fig 6.6). When the γ396-411 peptide was bound to Clf41(221-559), no difference in binding was seen with mAb6 (Fig. 6.7). Small but significant reductions in binding were seen for both mAbs36 and 47 ($p=0.01$). Both of these mAbs also bound less to the Clf41(221-559)-fibrinogen complex. The epitope for mAb6 seemed to become more exposed when bound to this Clf41(221-559)-fibrinogen complex. A dramatic 2.5-fold increase in binding occurred. However, when the γ396-411 peptide and whole fibrinogen were bound to Clf41(221-559) immobilized on an ELISA plate, the same data could not be generated (Fig. 6.8). The binding of mAbs 36 and 47 were affected less by whole fibrinogen and no induction of the mAb6 epitope by fibrinogen was seen.

6.3 Discussion

When a monoclonal antibody binds to a protein transferred to nitrocellulose from an SDS-PAGE gel, it may be reasonable to assume that it recognises a linear

Figure 6.6. Anti-Clf33(221-550) polyclonal IgG reactivity with Clf41(221-559) complexed with the fibrinogen C-terminal γ-chain or whole fibrinogen. Data shown is from a single ELISA well reading.

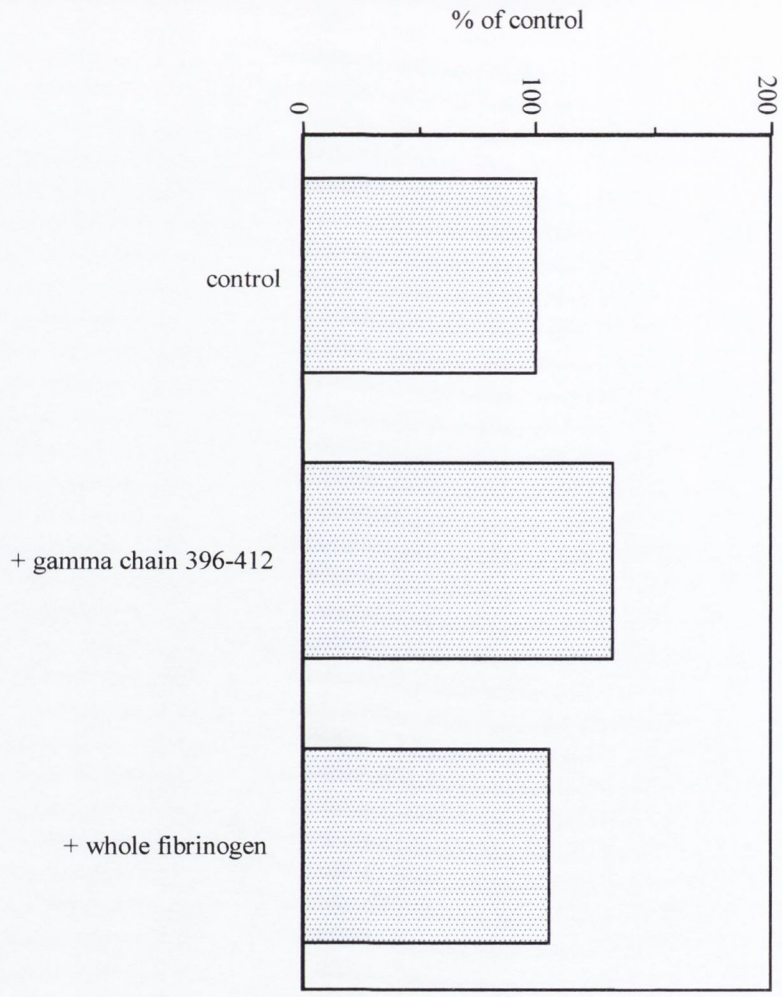

% of control
0
100
200
control
+ gamma chain 396-412
+ whole fibrinogen

Figure 6.7. Binding of mAbs 6, 36 and 47 to Clf41(221-559) complexed in solution before coating of ELISA wells with the fibrinogen C-terminal γ-chain or whole fibrinogen. Data points show the mean of triplicate ELISA wells from one representative experiment. Errors are the standard deviation from the mean.

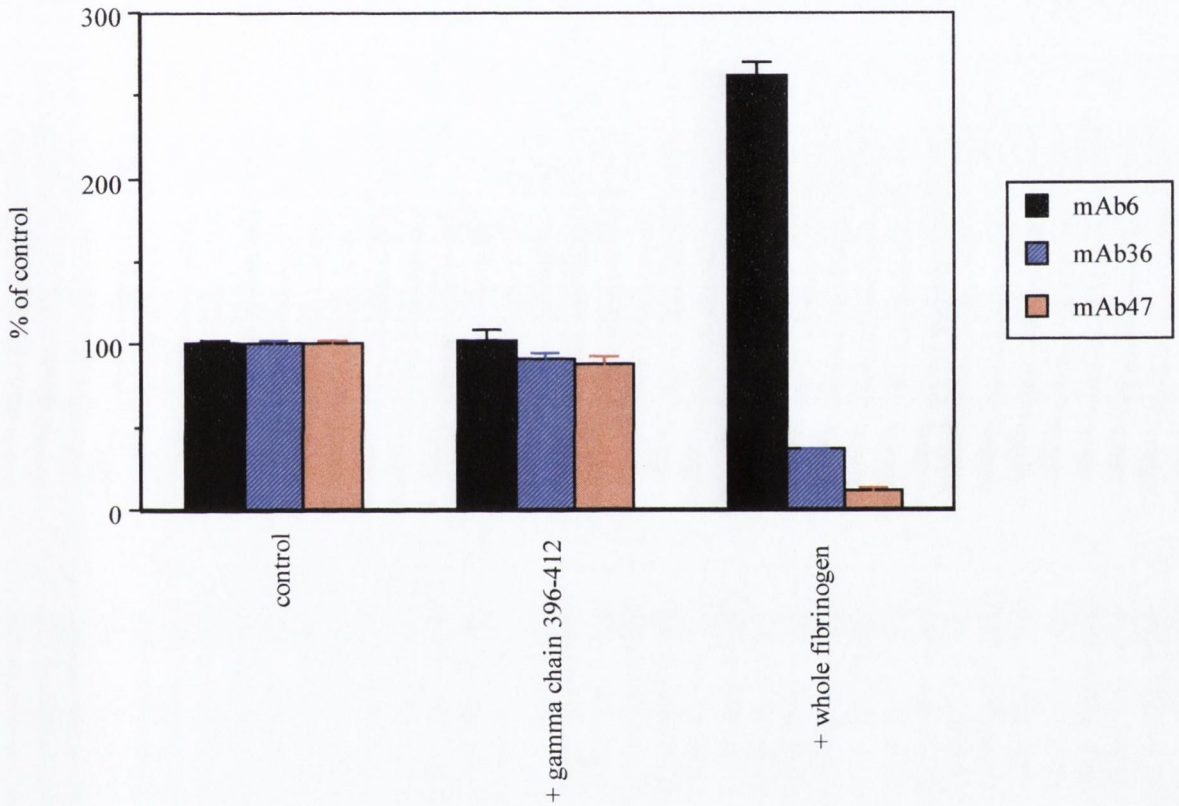
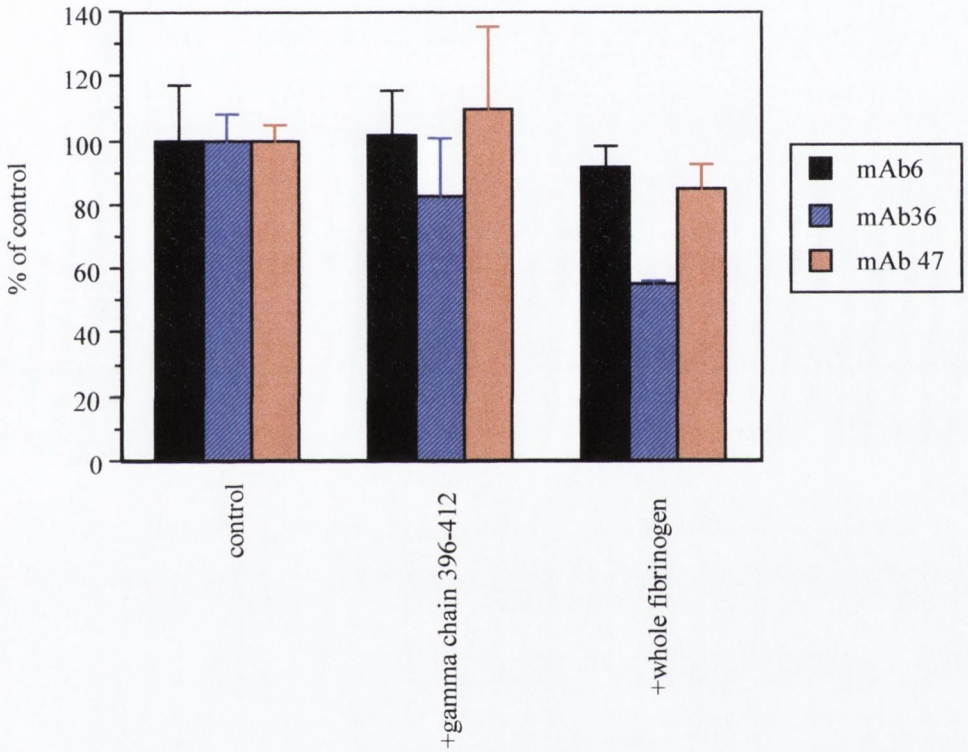

300
200
100
0
% of control
control
+ gamma chain 396-412
+ whole fibrinogen
mAb6
mAb36
mAb47

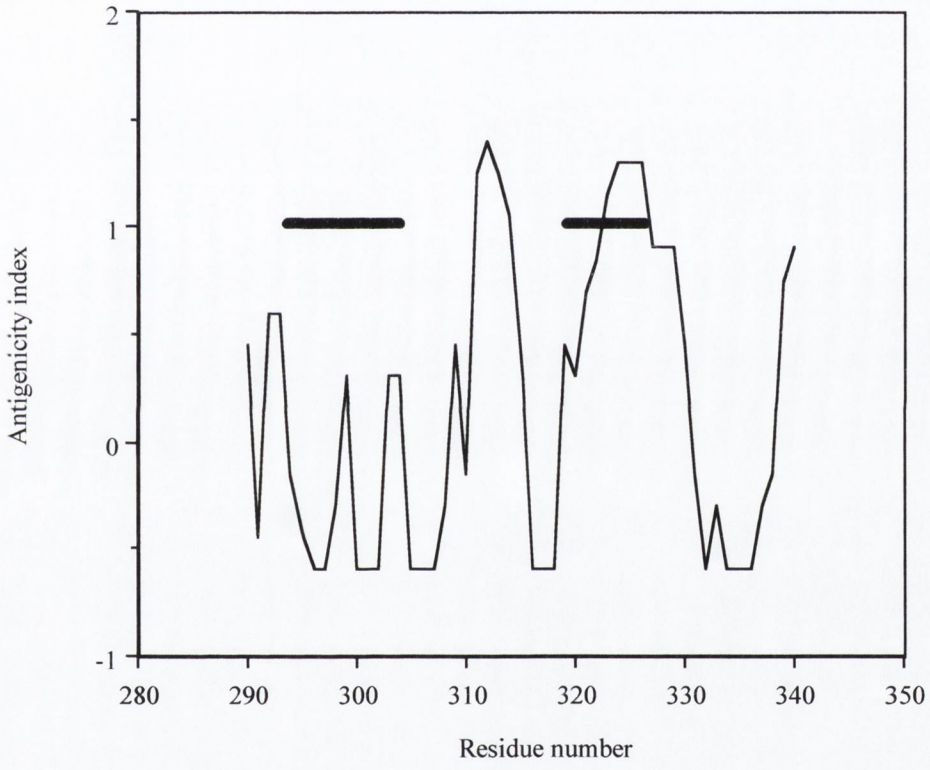
Figure 6.8. Effect of the fibrinogen C-terminal γ-chain and whole fibrinogen on mAbs 6, 36 and 47 binding to immobilized Clf41(221-559). Data points shown are the mean of triplicate determinations from one representative experiment. Error bars are the standard deviation from the mean.


140
120
100
80
60
40
20
0
% of control
control
+gamma chain 396-412
+whole fibrinogen
mAb6
mAb36
mAb 47

epitope (De Los Toyos *et. al.,* 1996). However, proteins that are denatured by SDS have the ability to refold to their native conformation when transferred to nitrocellulose in the presence of a transfer buffer. The Western immunoblotting data for the mAbs in this study suggest that the proteins were capable of renaturing when transferred. No recombinant molecule was recognised by a mAb on ELISA that was not recognised on a Western immunoblot. Therefore, the effect of using a denaturing gel before transfer was probably minimal. Polyclonal IgG raised against Clf33(221-550) bound to all recombinant deletion fragments, as it recognised multiple epitopes over the whole length of Clf33(221-550) and would not be largely affected in binding to these truncates (Table 6.1).

The epitopes recognised by mAbs 36 and 47 could be localised to the domain 221-424 of ClfA. The EF hand motif, which has been shown to have a major role in fibrinogen binding by ClfA is within this area of the protein. Both of these mAbs bind all of the truncates which contain the EF hand except 272-559. EF hands are flanked by α-helices, and are therefore conformational binding sites (Voet and Voet, 1990). These α-helices are present in ClfA flanking the residues 310-321 (Fig. 6.9). The recombinant truncate composed of residues 272-559 may not be capable of folding correctly to expose the epitope recognised by mAbs 36 and 47. A mAb may not be capable of binding to a truncate for two reasons. Either the epitope residues are present but are in an incorrect conformation for mAb recognition, or the residues of the epitope themselves are not present. The truncates composed of residues 23-308, 332-559, 272-559 are all negative for binding by mAbs 36 and 47. Both mAbs 36 and 47 show the same pattern of reactivity to the truncates used in the panel. However, because the combined inhibitory effect of these mAbs is synergistic, they cannot recognise the same epitope. These two antibodies possibly recognise closely linked conformational epitopes in or near to the EF hand. Residues 311-314 within the EF hand motif are predicted to be antigenic (Fig. 6.1). Also, there is another predicted site, within the α-helical segment flanking the C-terminal of the EF hand motif. The binding of an antibody at this site could disrupt the overall structure of the active site. Another alternative is that mAbs36 and 47 could bind elsewhere in 221-424 and exert long-range conformational effects on the EF hand rendering it incapable of interacting with fibrinogen. The residues of the MIDAS motif have a low but positive antigenicity index (0.450-0.600) as have the first three residues of the EVA/G motif sequence

Figure 6.9. Antigenicity profile for ClfA residues 290-340. Solid black lines show predicted α-helices in the vicinity of the EF hand motif (residues 310-321)


2
1
0
-1
Antigenicity index
280
290
300
310
320
330
340
350
Residue number

(0.450-0.600) where the G at residues 532 is part of a region of high antigenic potential with a value of 1.700. However, mAb6 cannot bind directly to this 531-539 epitope as its domain is located between residues 332-525.

The pattern of binding of mAb6 to recombinant truncates on Western immunoblot is completely different to that seen for mAbs 36 and 47. This data would suggest that this mAb recognised an area of the protein distinct from that of mAbs 36 and 47. There is no possibility that mAb6 binds to the EF hand motif as this antibody was raised against Clf42(332-559), a truncate which does not contain this motif. The epitope recognised by this mAb is extremely sensitive to deletion from the C-terminus. For example, mAb6 reacts with 332-550, but not with a truncate containing residues 221-484. The epitope recognised by this antibody could not be localised any further than 332-525. This would suggest the epitope defined by mAb6 is highly conformational. Also, an attempt was made to map the epitope recognised by mAb47 using a biopanning technique with a random library of 12-mer peptides in an M13 based display system (Wann, Personal communication). No meaningful data could be generated by this method, further suggesting that the epitopes recognised by mAb47, and possibly the other two inhibitory mAbs characterized are conformational.

Anti-Clf33(221-550) IgG bound to two of the peptides in the series spanning residues 333-557. The first of these, within residues 373-397 is in the domain overlapping the regions defined by mAbs 36/47 and mAb6. The second linear epitope detected was within residues 533-557. Although it is clear that polyclonal IgG recognised linear epitopes in these regions, they may not be important ones in mediating the inhibitory activity demonstrated by this polyclonal antibody. Previous experiments have shown that these peptides individually or indeed all peptides combined together, were not able to neutralise the inhibitory effects of anti-Clf33(221-550) polyclonal IgG (McDevitt, Personal communication).

Neither mAb 36 nor 47 bind to solid-phase bound 25-mer peptides corresponding to residues 332-424 of ClfA, only the first five of which are relevant to mAbs 36 and 47. Also, no binding to solid-phase ClfA peptides was seen by mAb6. This result is not surprising if the epitope bound by mAb6 is conformational. In fact, all of the inhibitory mAbs described in this study may recognise conformational epitopes rather than linear ones. In this case, even if peptides with sufficient overlap were generated, the mAbs would not recognise a linear peptide. A series of 90 pentadecapeptides overlapping by 13 residues corresponding to the Rac-1 protein were

generated (Joseph and Pick, 1995). This is a molecule that activates the superoxide generating NADPH oxidase of phagocytes. It was found that the peptides containing important motifs for the inhibition of protein activity must be on the end of a peptide in order to function. Solid-phase peptides may also be constrained when bound to plastic. No binding of either mAb was detected to EF hand peptide. This is not surprising as the EF hand co-ordinating residues are merely the loop within a helix-loop-helix structural ligand-binding site (Fig. 6.10, Voet and Voet, 1990). However, mAbs 36 and 47 were affected in binding to Clf41(221-559) by preincubation with the soluble EF hand peptide indicating that it may form part of the epitopes recognised by these mAbs (Fig. 6.4).

Both mAbs 36 and 47 showed similar binding patterns to ClfA site-directed mutant proteins on ELISA. These mAbs were both affected in their binding to the EF hand quadruple mutant, where they were unaffected in their reaction with a double MIDAS mutant. However, a difference was seen on binding of these two mAbs to another C-terminal region A quadruple site-specific recombinant, SM4. This is an indication that these mAbs are recognising distinct epitopes, as mAb47 is unaffected in binding to the SM4 mutant protein.

Binding of mAbs 36 and 47 to Clf41(221-559) complexed with whole fibrinogen was reduced. This effect was only partial as 63% and 81% reductions were observed for mAbs 36 and 47 respectively. The fibrinogen molecule may sterically affect recognition of the epitopes corresponding to these mAbs. The binding of fibrinogen may also not directly block the binding of the mAbs to the protein, but alter the conformation of Clf41(221-559) such that mAbs 36 and 47 cannot bind as avidly to the molecule. A much smaller but repeatable reduction in binding of both mAbs (8% and 12% reduction in binding of mAbs 36 and 47 respectively) was also seen when Clf41(221-559) was complexed with the fibrinogen C-terminal γ-chain peptide. This further indicates that the EF hand has some interaction with the epitopes defined by these mAbs. This result favours a model where mAbs 36 and 47 do not bind directly to EF hand, but a site that affects the activity of this site either by steric hindrance of fibrinogen binding or conformational change.

The pattern of mAb6 binding was again different to that of the other mAbs. This antibody was not affected in its binding to the mutant protein containing the quadruple mutation in the EF hand. This data is in agreement with mAb6 showing a

positive reactivity with Clf51, the EF hand deletion mutant, on Western immunoblots (Table 6.1). In fact, mAb6 showed increased binding to this mutant protein. Perhaps the mutation alters the conformation of the protein such that the epitope recognised by mAb6 is more exposed. However, a double mutation in the MIDAS motif residues D443 and S445 affects mAb6 binding significantly. Also, the C-terminal site-directed quadruple mutation at residues E527, V528, A529 and G532 appeared to alter mAb6 binding dramatically.

mAb6 binding to the Clf41(221-559)-γ396-412 complex is not affected. This is not surprising if this mAb binds directly to the MIDAS motif or another site nearby in the C-terminus. This mAb is a useful probe to demonstrate that no major conformational change occurs in the C-terminus on γ-chain binding to the EF hand. A dramatic increase in binding is seen when mAb6 reacts with the Clf41(221-559)-fibrinogen complex. The epitope recognised by mAb6 must be partially concealed in Clf41(221-559), but fully exposed in Clf42(332-559), the molecule which it was raised against. This mAb bound 33% less avidly to Clf41(221-559) than Clf42(332-559) on ELISA. The conformational state of Clf41(221-559) when bound to fibrinogen must be similar to that of the Clf42(332-559) recombinant protein. Perhaps the deletion of residues 221-331 prevents a folding event necessary for ligand binding. As fibrinogen binding to Clf41(221-559) causes dramatic increase in mAb6 reactivity, it therefore must not bind directly to the MIDAS motif, as fibrinogen would occupy the MIDAS binding site mAb6 most likely mediates its inhibitory effect by altering conformation of the MIDAS motif. Converse to this, the fibrinogen binding event must alter the conformation of Clf41(221-559) so that the mAb6 epitope is much more accessible. This result is also in agreement with the increased binding seen for mAb6 with the EF hand quadruple mutant protein.

It is important to note that the ELISA data for the mAbs and the Clf41(221-559) complexes could not be reproduced when whole fibrinogen or the γ-chain peptide was added to solid-phase Clf41(221-559) bound to ELISA wells before complex formation (Fig. 6.10). This demonstrates the importance of Clf41(221-559) being in the soluble phase in order to fold correctly when binding to fibrinogen or its C-terminal γ-chain.

Conformational changes such as that described for soluble Clf41(221-559) in this study are found to occur in other systems also. The binding of ferrichrome to the

ferrichrome-iron receptor of *E. coli* K-12 was found to induce a localised structural change in a periplasmically exposed loop of FhuA (Moeck *et. al.,* 1996). This periplasmic domain was defined by certain mAbs that could discriminate between free FhuA and FhuA bound to ferrichrome. Monoclonal antibodies have been identified which markedly enhance the binding of fibronectin to recombinant *Streptococcus dysgalactiae* FnbA fragments (Speziale *et. al.,* 1996). It was proposed that these enhancing mAbs changed the unordered structure of the unoccupied ligand-binding unit to a conformation normally induced by fibronectin binding. Pure mAb did not recognise the antigen by itself, and the presence of fibronectin was necessary for the immunological interaction between the antibody and the epitope. The epitope was located to a region just N-terminal of the ligand binding site. Indeed it has been shown that conformational changes in the FnbA occur on ligand binding (House-Pompeo *et. al.,* 1996). On addition of fibronectin N-terminus to recombinant FNBD-A, there was a large change in the resulting far-UV CD difference spectra.

A conformational change has been shown to occur on Clf41(221-559) and also full length region A, Clf40(40-559), binding in solution to the C-terminal γ-chain of fibrinogen (O'Connell, Personal communication). Also, Clf33(221-550) and Clf40(40-559) are currently being processed in order to obtain crystal structure data for these molecules. When the results become available, the mAb data presented in this chapter will more than likely be complemented by the structural information for the ligand-binding domain of ClfA.

CHAPTER 7

GENERAL DISCUSSION

This work set out to investigate whether it was possible to generate monoclonal antibodies to the ClfA protein of *S. aureus*, and to use these antibodies in order to characterize the interaction between ClfA and fibrinogen. It was shown that from a panel of eleven mAbs generated against Clf33(221-550), the minimum fibrinogen-binding truncate, two of these had inhibitory activity when purified. These were mAbs 36 and 47. Also, out of thirty-eight mAbs isolated against Clf42(332-559), a non-fibrinogen binding protein, six were identified which blocked the ClfA-fibrinogen interaction. Unfortunately, only one of the hybridoma clones which secreted these anti-Clf42(332-559) mAbs, mAb6, was stable enough to merit further characterization. The properties of these three mAbs as determined in this study are summarized in Table 7.1. All of these antibodies blocked bacterial clumping in soluble fibrinogen. However, this effect was relatively weak for each mAb in this assay. mAbs 6 and 47 also had detectable blocking activity in a bacterial adherence assay to immobilized fibrinogen. No mAb was identified which showed potent inhibition as compared to anti-Clf33(221-550) polyclonal IgG.

This polyclonal IgG and all three mAbs showed specificity for ClfA with respect to other members of the SD-repeat multigene family of proteins from *S. aureus*. However, polyclonal IgG seemed to cross-react with another surface component of whole *S. aureus* cells other than ClfA, as it bound to ClfA-mutant cells. It is possible that anti-Clf33(221-550) polyclonal IgG is reactive with the FnBPA and FnBPB as the A domains of these proteins share 20-30% identical residues (McDevitt *et. al.,* 1994). Most of this similarity occurs in the region between ClfA residues 200-500. Recently, the recombinant A domain of FnBP was found to be capable of binding to fibrinogen (Wann, Personal communication).

It is important to note the fact that it was possible to generate blocking polyclonal and monoclonal antibodies against ClfA, as it has proved extremely difficult to isolate antibodies which block the FnBP-fibronectin interaction (Ciborowski *et. al.,* 1992, Rozalska *et. al.,* 1994, Speziale *et. al.,* 1996) until a recent study which isolated some polyclonal antibodies generated against recombinant fibronectin-binding domains were found to have weak inhibitory activity. More rigorous screening of a larger number of hybridoma clones may have identified more potent mAbs. Out of 100 mAbs screened for in vivo neutralization of tetanus toxin, only six were found to have protective properties (Lang *et. al.,* 1993). Only one of these provided protection for more than 28 days in a standard potency assay, while the remaining five merely resulted

TABLE. 7.1. Summary of the properties of mAb6, 36 and 47.

	mAb6	mAb36	mAb47
raised against recombinant truncate	332-559	221-550	221-550
reactivity with other SD-repeat proteins	-	-	-
inhibition of bacterial clumping in Fg[a]	+	+	+
inhibition of bacterial adherence to Fg	+	ND[b]	+
inhibition of Fg-Clf41(221-559) interaction	+	+	+
inhibition of bacterial attachment to plasma clots	NP[c]	+	+
protective effect in virulence model	NP	NC[d]	NC
epitope location	332-525	221-424	221-424
site-directed mutation affecting binding	MIDAS/EVA	EF/EVA	EF
binding to Clf41(221-559) complexes	LIBS[e] with Clf41-Fg	decrease with Clf41-Fg and Clf41-γ chain	decrease with Clf41-Fg and Clf41-γ chain

[a] Fg; fibrinogen.

[b] ND; the inhibitory activity of mAb36 was not detectable in the bacterial attachment assay.

[c] NP; experiment was not performed.

[d] NC; it is not clear from the data of the mouse infection model whether or not mAbs 36 and 47 assayed in combination showed any protective effect.

[e] LIBS; ligand-induced binding site.

in delayed death of the mice. The large number of different epitopes recognized by polyclonal antibody preparations normally ensures the presence of protective antibodies, which are usually a small subpopulation. Replacement by mAbs is only feasible only if one or a small number of mAbs recognize such protective epitopes, and can confer the same level of protection achieved by polyclonal antibody.

The three inhibitory mAbs identified were then purified and subject to more exact assays to determine their potency in inhibiting the ClfA-fibrinogen interaction. All mAbs inhibited bacterial clumping in soluble fibrinogen in the range 125-250μg/ml. Only at high IgG concentration did mAb6 demonstrate the ability to block bacterial attachment to solid-phase fibrinogen, while mAb47 showed a slightly more potent effect. The blocking activity of mAb36 could not be detectable in this assay when assayed alone, presumably because it has a weaker inhibitory activity than the other two mAbs individually. However, another ELISA where the mAbs blocked a ClfA-fibrinogen protein-protein interaction proved to be more sensitive, as mAb36 activity was demonstrated in this assay. mAbs 6 and 47 also blocked the binding of whole fibrinogen to immobilized Clf41(221-559). When the mAbs were assayed in pairwise combinations, a synergistic blocking effect was observed when mAbs 36 and 47 were assayed together. This synergism occurred in both the soluble clumping assay and the bacterial adherence assay, but not in the inhibition of fibrinogen binding to immobilized Clf41(221-559). The results of this assay together with other experiments described later in the study suggest that Clf41(221-559) is not capable of conformational change when immobilised to polystyrene wells. Synergism with mAbs has been observed in other instances with mAbs raised against other bacterial proteins. Two mAbs produced against TSST-1 inhibited the binding of this protein to HLA-DR with a greater potency than additive inhibitions (Darji *et. al.,* 1996). Also, in a standard tetanus toxin neutralization assay in mice, two human mAbs showed a synergistic protective effect (Lang *et. al.,* 1993).

The bacterial adherence to platelet-fibrin clots in vitro can be considered to be a semi-in vivo adherence model. It involves bacterial adherence to a complex fibrinogen-containing substrate rather than to purified fibrinogen, and may mimic wound or foreign body infection more closely. It has been shown that a ClfA$^-$ mutant has a statistically significantly reduced level of adherence to platelet-fibrin clots in vitro both in this study and in a previous one (Moreillon *et. al.,* 1995). Purified Clf33(221-550) did not reduce

S. aureus adherence to the clots, presumably because it could not bind to the fibrinogen present within this environment. Anti-Clf33(221-550) polyclonal IgG reduced bacterial adherence six-fold while 20μg/ml of mAbs 36 and 47 combined blocked *S. aureus* cells binding by approximately 40%.

It was unfortunate that in vivo data could not be generated as part of this study. Perhaps the murine renal infection and septicaemia model used would not have been relevant to the role of ClfA in *S. aureus* infection. It is known that ClfA may have a role in the pathogenesis of infective endocarditis (Moreillon *et. al.,* 1995). Also, another recent study has shown some promising preliminary data. Active immunization with Clf40(40-559) is protective against intravenous challenge with *S. aureus* strain MRSA 601 (Patti, Personal communication). Using the same model, passive immunization with IgG raised against Clf30(332-550) also protected against *S. aureus* infection. This model offers greater control over the number of bacteria in the bloodstream so that protective effects of anti-ClfA antibodies will be seen more readily.

The domains of ClfA recognised by mAbs 6, 36 and 47 were then localised using Western immunoblotting of recombinant ClfA truncates. mAbs 36 and 47 were found to bind within a 203 amino acid section consisting of residues 221-424. Although many truncates were used with a varied selection of ClfA residues, the fact that the domains recognised by these mAbs could not be localized any further by this method suggests that the epitopes are probably conformational in nature. None of the three inhibitory mAbs reacted with immobilized 25-mer peptides overlapping by five amino acids corresponding to residues 333-557 of ClfA. Although the mAb epitope residues may have been present in the peptide sequences, immobilization may have affected the ability of the mAbs to bind the peptides. Anti-Clf33(221-550) polyclonal IgG reacted with two of these peptides, 373-397 and 533-557. These peptides contain linear epitopes that are probably not important to the blocking activity of the polyclonal IgG, as it has been previously shown that these peptides in solution do not neutralize the blocking activity of anti-ClfA antibodies (McDevitt, Personal communication).

The fact that mAbs 36 and 47 localized to residues 221-424, the inhibitory effect could not be due to direct binding to the MIDAS-like motif (443-447) or the EVA motif (526-532), but they could potentially bind directly to the EF hand (310-321). mAbs 36 and 47 were indeed affected in their binding to a recombinant protein which contained a quadruple site-directed mutation in the EF hand. Neither of these mAbs bound to

another recombinant region A protein on Western immunoblots that contained a deletion in the EF hand residues from 310-321. Also, when the fibrinogen C-terminal γ-chain was bound to Clf41(221-559), small but repeatable and significant reductions in mAbs 36 and 47 binding to this complex was seen. Both antibodies also showed much reduced binding to Clf41(221-559) complexed with whole fibrinogen. Also, a soluble peptide corresponding to the EF hand residues 310-321 partially inhibited mAbs 36 and 47 binding to Clf41(221-559). All this data taken together suggests that the EF hand may constitute at least a part of the epitopes recognised by mAbs 36 and 47. As these mAbs inhibit bacterial interactions with fibrinogen synergistically, they cannot recognise the same epitope. However, the epitopes recognised by the two mAbs could potentially overlap each other, as they exhibit almost identical reactivity patterns. The only distinctions between these two mAbs is that mAb36 seems to have a weaker blocking capability than mAb47, and mAb36 binding is affected by a quadruple site-directed mutation in the extreme C-terminus at residues 526, 527, 528 and 532. This mutation probably causes long-range conformational effects in the epitope recognised by mAb36, but this mutation does not seem to affect mAb47 binding.

Although mAb6 also demonstrated the ability to block the ClfA-fibrinogen interaction, it does not recognise the same epitope as mAbs 36 and 47. Western immunoblotting data localised the mAb6 epitope to between residues 332-525. This antibody was raised against Clf42(332-559), the non-fibrinogen binding truncate which has the ability to neutralise the blocking effect of anti-Clf33(221-550) polyclonal serum (McDevitt *et. al.,* 1994). It is not possible that this antibody mediates its blocking effect by direct binding to the EF hand (310-321) or EVA motif (526-532). This mAb is not affected in binding to Clf51(Δ310-321) on Western immunoblots and retains the ability to bind to the quadruple site-directed EF hand mutant. However, mAb6 demonstrated reduced binding to the mutants in the MIDAS motif and the EVA motif. This antibody did not show reduced binding to Clf41(221-559) complexed with the fibrinogen C-terminal γ-chain or whole fibrinogen. In fact, the epitope defined by mAb6 is much more exposed when Clf41(221-559) is complexed with whole fibrinogen. This would suggest that the Clf42(332-559) truncate exists in a conformation similar to that of ClfA when it is bound to fibrinogen.

Polyclonal antibodies raised against recombinant ClfA proteins were much more potent than the best synergistic mAbs identified in this study. However, ClfA seems to

be capable of generating a favourable immune response at least in mice and rabbits. Preliminary data using patient serum also suggests that an immune response occurs during human disease (Hook, Personal communication). It was also possible to detect anti-ClfA LIBS antibodies in patient serum, unlike the immune response directed against Cna. In general, lower titres were obtained against Cna as opposed to ClfA, and no LIBS antibodies against Cna were evident. Therefore, ClfA may be an important candidate for inclusion in an anti-staphylococcal vaccine.

Other *S. aureus* antigens have shown promise as potential vaccine components. Immunization with RNAIII-activating protein, an *agr*-encoded protein involved in regulating the expression of staphylococcal exoproteins, is protective in an experimental model of cutaneous infection (Balaban *et. al.,* 1998). Also, a capsular polysaccharide-protein conjugate vaccine has produced improved phagocytosis in vitro and improved survival in experimental models of staphylococcal infection, including endocarditis (Fattom *et. al.,* 1996, Lee *et. al.,* 1997). In fact, capsule-protein conjugate vaccines have also stimulated an antibody response in human volunteers (Fattom *et. al.,* 1993, 1996). These antibodies showed opsonic activity in vitro phagocytosis tests, and conferred passive immunity to mice and rats in animal models of infection. A segment of the fibronectin-binding protein was found to stimulate protective immunity against rat endocarditis and mouse and bovine mastitis (Nelson *et. al.,* 1992, Rozalska and Wadstrom, 1993, Schennings *et. al.,* 1993). Small molecule inhibitors may also block adherence of *S. aureus* to wounds and conditioned biomaterials and in this manner prevent disease. Synthetic peptides corresponding to the ligand-binding domains of FnBP and a peptide corresponding to a section of the Cna protein inhibited bacterial binding to host proteins in vitro (Raja *et. al.,* 1990, Patti *et. al.,* 1995). Perhaps one of the ClfA recombinant truncates or antibodies derived from immunization with the same proteins will be used in the future in order to treat staphylococcal infection.

REFERENCES

Abdelnour, A., S. Arvidson, T. Bremnell, C. Ryden, and A. Tarkowski. 1993. The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. Infect. Immun. **61:**3879-3885

Alber, G., D. Hammer, and B. Fleischer. 1990. Relationship between enterotoxic- and T lymphocyte-stimulating activity of staphylococcal enterotoxin B. J. Immunol. **144:**4501

Albus, A., R.D. Arbeit, and J.C. Lee. 1991. Virulence of *Staphylococcus aureus* mutants altered in type 5 capsule production. Infect. Immun. **59:**1008-1014

Altieri, D.C., J Plescia and E.F. Plow. 1993. The structural motif glycine190-valine 202 of the fibrinogen γ chain interacts with CD11b/CD18 integrin ($\alpha_M\beta_2$, Mac-1) and promotes leucocyte adhesion. J. Biol. Chem. **268:**1847-1853

Amman, E, and J. Brosius. 1985. ATG vectors for regulated high level expression of cloned genes in *Escherichia coli*. Gene **40:**183-190

Angrist, A.A, and M. Oka. 1963. Pathogenesis of bacterial endocarditis. J. Amer. Med. Assoc. **4:**249-252

Baddour, L.M., C. Lowrance, A. Albus, J.H. Lowrance, S.K. Anderson, and J.C. Lee.. 1992. *Staphylococcus aureus* microcapsule attenuates bacterial virulence in a rat model of experimental endocarditis. J. Infect. Dis. **165:**749-753

Baddour, L.M. 1994. Virulence factors among gram-positive bacteria in experimental endocarditis. Infect. Immun. **62:**2143-2148

Balaban, N. and R.P. Novick. Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **92:**1619-1623.

Balaban, N., Goldkorn, T., and Nhan, R.T. 1998. Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*. Science **280:**438-440

Balwit, J.M., P. van Langeveld, J.M. Vann, and R.A. Proctor. 1994. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J. Infect. Dis. **170:**1033-1037

Barenkamp, S.J., and J.W. St. Geme III. 1996. Identification of surface-exposed B-cell epitopes on high molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae*. Infect. Immun. **64:**3032-3037

Barioch, A. 1991. PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. **19:**2241-2245

Bayer, A.S., P.M. Sullam, M. Ramos, C. Li, A.L. Cheung, and M.R. Yeaman. 1995. *Staphylococcus aureus* induces platelet aggregation via a fibrinogen-dependent mechanism which is independent of principal platelet glycoprotein IIb/IIIa fibrinogen-binding domains. Infect. Immun. **63:**3634-3641

Bayer, M.G., J.H. Heinrichs, and A.L. Cheung. 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus.* J. Bacteriol. **178:**4563-4570

Begley, S. 1994. The end of antibiotics. Newsweek, March 28, pp 46-51

Bhakdi, S., M. Muhly, and R. Fussle. 1984. Correlation between toxin binding and hemolytic activity in membrane damage by staphylococcal α-toxin. Infect. Immun. **46:**318

Bhakdi, S., and J. Tranum-Jensen. 1991. Alpha-toxin of *Staphylococcus aureus.* Microbiol. Rev. **55:**733-751

Bobmer, M., M.A. Fournel, and L.B. Hinshaw. 1993. Preclinical review of anti-tumor necrosis factor monoclonal antibodies. Crit. Care Med. **21:**S441-446

Boden, M.K., and J.I. Flock. 1989. Fibrinogen-binding protein/clumping factor from *Staphylococcus aureus.* Infect. Immun. **57:**2358-2363

Boden, M.K., and J.I. Flock. 1994. Cloning and characterization of a gene for a 19kDa fibrinogen-binding protein from *Staphylococcus aureus.* Mol. Microbiol. **12:**599-606

Boden, M.K., and J.I. Flock. 1995. Incidence of the highly conserved fib gene and expression of the fibrinogen-binding (Fib) protein among clinical isolates of *Staphylococcus aureus.* J. Clin. Microbiol. **33:**2347-2352

Bohach, G.A., M.M. Dinges, D.T. Mitchell, D.H. Ohlendorf, and P.M. Schlievert. 1997. Ch. 4 Exotoxins pp83-111 *In* The staphylococci in human disease. Crossley, K.B., and Archer, G.L. (eds). Churchill Livingstone, New York.

Bohach, G.A., D.J. Fast, R.D. Nelson, and P.M. Schleivert. 1990. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. CRC Cric. Rev. Microbiol. **17:**251

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254

Casadevall, A. 1996. Antibody-based therapies for emerging infectious diseases. Emerging Infectious Diseases **2:**200-208

Cazaubon, S. 1988. Anti-HV3 peptide antibodies as probes for conformational changes in immunoglobulins. J. Immunol. Meth. **114:**13-20

Chan, P.F., and S.J. Foster. 1998. Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. J. Bacteriol. **180:**6232-6241

Chatwal, G.S., G. Albohn, and H. Blobel. 1987. Interaction between fibronectin and purified staphylococcal clumping factor. FEMS Microbiol. Lett. **44:**147-151

Chen, F., G.M. Kuziemko, P. Amersdorfer, C. Wong, J.D. Marks, and R.C. Stevens. 1997. Antibody mapping to domains of botulinum neurotoxin serotype A in the complexed and uncomplexed forms. Infect. Immun. **65:**1626-1630

Cheung, A.L., K.J. Eberhardt, E. Chung, M.R. Yeaman, P.M. Sullam, M. Ramos, and A.S. Bayer. 1992. Diminished virulence of a *sar*$^-$/*agr*$^-$ mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. J. Clin. Invest. **94:**1815-1822

Cheung, A.L., M.R. Yeaman, P.M. Sullam, M.D. Witt and A.S. Bayer. 1994. Role of the *sar* locus of *Staphylococcus aureus* in induction of endocarditis in rabbits. Infect. Immun. **62:**1719-1725

Cheung, A.L. and P. Ying. 1994. Regulation of α- and β-hemolysins by the *sar* locus of *Staphylococcus aureus*. J. Bacteriol. **176:**580-585

Cheung, A.L., K.J. Eberhardt, and E. Chung. 1994b. Diminished virulence of a *sar*$^-$/*agr*$^-$ mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. J. Clin. Invest. **94:**1815-1822

Cheung, A.I., S.J. Projan, R.E. Edelstein, and V.A. Fischetti. 1995. Cloning, expression and nucleotide sequence of a *Staphylococcus aureus* gene (*fbpA*) encoding a fibrinogen-binding protein. Infect. Immun. **63:**1914-1920

Cheung, A.L., K. Eberhardt, and J.H. Heinrichs. 1997. Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. Infect. Immun. **65:**2243-2249

Chia, J.S., R.H. Lin, S.W. Lin, J.Y. Chen, and C.S. Yang. 1993. Inhibition of the glucosyltransferase activities of *Streptococcus mutans* by a monoclonal antibody to a subsequence peptide. Infect. Immun. **61:**4689-4695

Ciborowski, P., J.-I. Flock, and T. Wadstrom. 1992. Immunological response to a *Staphylococcus aureus* fibronectin-binding protein. J. Med. Microbiol. **37:**376-381

Coleman, D.C., J.P. Arbuthnott, H.M. Pomeroy, and H. Birbeck. 1986. Cloning and expression in *Escherichia coli* and *Staphylococcus aureus* of the beta-lysin determinant from *Staphylococcus aureus*: evidence that bacteriophage conversion of beta-lysin activity is caused by insertional inactivation of the beta-lysin determinant. Microb. Pathol. **1:**549-564

Collawn, J.F., C.J.A. Wallace, A.E.I. Proudfoot, and Y. Patterson. 1988. Monoclonal antibodies as probes of conformational change in protein-engineered cytochrome c. J. Biol. Chem. **263:**8625-8634

Comstock L.E., E. Fikrig, R.J. Shoberg, R.A. Flavell, and D.D. Thomas. 1993. A monoclonal antibody to OspA inhibits association of *Borrelia burgdorferi* with human endothelial cells. Infect. Immun. **61:**423-431

Coombs, R.R.A. 1987. Harnessing the red cell for immunoassays. Med. Lab. Sci. **44:**66-72

Courcol, R.J., D. Trivier, M.C. Bissinger, G.R. Martin, and M.R.W. Brown. 1997. Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. Infect. Immun. **65:**1944-1948

Craig, A.G., R. Pinches, S. Khan, D.J. Roberts, G.D.H. Turner, C.I. Newbold, and A.R. Berendt. 1997. Failure to block adhesion of *Plasmodium falciparum*-infected erythrocytes to ICAM-1 with soluble ICAM-1. Infect. Immun. **65:**4580-4585

Crawford, J., and C. Russell. 1986. Comparative adhesion of seven species of streptococci isolated from the blood of patients with sub-acute bacterial endocarditis to fibrin-platelet clots in vitro. J. Appl. Bacteriol. **60:**127-133

Cribier, B., G. Prevost, P. Coupie, V. Finck-Barbancon, E. Grosshans, and Y. Piemont. 1992. Staphylococcus aureus leukocidin: a new virulence factor in cutaneous infections? An epidemiological and experimental study. Dermatology **185:**175-180

Darji, A., K. Niebuhr, M. Hense, J. Wehland, T. Chakraborty, and S. Weiss. 1996. Neutralising monoclonal antibodies against listeriolysin: mapping of epitopes involved in pore formation. Infect. Immun. **64:**2356-2358

De Los Toyos, J.R., F.J. Mendez, J.F. Aparicio, F. Vazquez, M. Del Mar Garcia Suarez, A. Fleites, C. Hardisson, P.J. Morgan, P.W. Andrew, and T.J. Mitchell. 1996. Functional analysis of pneumolysin by use of monoclonal antibodies. Infect. Immun. **64:**480-484

Doolittle, R.F. 1984. Fibrinogen and Fibrin. Ann. Rev. Biochem. **53:**195-229

Dorman, C.J. 1994. Virulence gene regulatory networks. *In* Genetics of bacterial virulence. Blackwell Scientific Publications

D'Souza, S.E., M.H. Ginsberg, T.A. Burke and E.F. Plow. 1990. The ligand-binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its a subunit. J. Biol. Chem. **265:**3440-3446

D'Souza, S.E., M.H. Ginsberg, G.R. Matseuda and E.F. Plow. 1991. A discrete sequence in platelet integrin is involved in ligand recognition. Nature **350:**66-68

Duthie, E.S. 1954. Evidence for two forms of staphylococcal coagulase. J. Gen. Microbiol. **10:**427-436

Duthie, E.S., and L.L. Lorenz. 1952. Staphylococcal coagulase: mode of action and antigenicity. J. Gen. Microbiol. **6:**95-107

Fattom, A.I., R. Schneerson, S.C. Szu, W.F. Vann, J. Shiloach, W.W. Karakawa, and J.B. Robbins. 1990. Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **58:**2367-2374

Fattom, A.I., R. Schneerson, D.C. Watson, W.W. Karakawa, D. Fitzgerald, I. Pastan, X. Li, J. Shiloach, D.A. Bryla, and J.B. Robbins. 1993. Laboratory and clinical evaluation of conjugate vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides bound to *Pseudomonas aeruginosa* recombinant exotoxin A. Infect. Immun. **61:**1023-1032

Fattom, A.I., Sarwar, J., Ortiz, A., and Naso, R. 1996. A *Staphylococcus aureus* capsular polysaccharide (CP) vaccine and CP-specific antibodies protect mice against bacterial challenge. Infect. Immun. **64:**1659-1665

Fernandez-Guerrero, M.L., C. Verdejo, J. Azofra, and M. de Gorgolas. 1995. Hospital-acquired infectious endocarditis not associated with cardiac surgery: an emerging problem. Clin. Infect. Dis. **20:**16-23

Finck-Barbancon, V., G. Prevost, and Y. Piemont. 1991. Improved purification of leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains. Res. Microbiol. **142:**75-85

Flock, J.-I, S.A. Heinz, A. Heimdahl, and T. Schennings. 1996. Reconsideration of the role of fibronectin binding in endocarditis caused by *Staphylococcus aureus*. Infect. Immun. **64:**1876-1878

Foster, T.J., M. O'Reilly, and P. Phonimdaeng. 1990. p403. Genetic studies of virulence factors of *Staphylococcus aureus*. Properties of coagulase and γ-toxin and the role of α-toxin, β-toxin and protein A in the pathogenesis of *S.aureus* infections. *In* Novick, R.P. (ed.) Molecular Biology of the Staphylococci, VCH, New York.

Foster, T.J., and M. Hook. 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends. Microbiol. **6:**484-488

Freer, J.H., and T.H. Birbeck. 1982. Possible conformation of delta-lysin, a membrane-damaging peptide of *Staphylococcus aureus*. J. Theor. Biol. **94:**535-540

Freer, J.H. 1988. Toxins as virulence factors of gram positive pathogenic bacteria of veterinary importance. pp264-288 *In* Roth, J.A. (ed.) Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington

Frost, P., L.S. Havarstein, B. Lygren, S. Stahl, C. Endresen, and K.E. Christie. 1995. Mapping of neutralisation epitopes on infectious pancreatic necrosis viruses. J. Gen. Virol. **76:**1165-1172

Gale, C., D. Finkel, N. Tao, M. Meinke, M. Mc Clellan, J. Olson, K. Kendrick, and M. Hostetter. 1996. Cloning and expression of a gene encoding an integrin-like protein in *Candida albicans*. Proc. Natl. Acad. Sci. USA **93:**357-361

Gaskill, M.E. and S.A. Khan. 1988. Regulation of enterotoxin B gene in *Staphylococcus aureus*. J. Biol. Chem. **263:**6276-6280

Gillaspy, A.F., S.G. Hickmon, R.A. Skinner, J.R. Thomas, C.L. Nelson, and M.S. Smeltzer. 1995. Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. Infect. Immun. **63:**3373-3380

Glenny, A.T., and N.F. Stevens. 1935. Staphylococcal toxins and antitoxins. J. Pathol. Bacteriol. **40:**201

Godinot, C., M. Moradi-Ameli, and D.C. Gautheron. 1986. Monoclonal antibodies to F1-ATPase subunits as probes of structure, conformation and functions of isolated or membrane bound F1. Methods in Enzymology **126:**761-769

Goldberg, M.E. 1991. Investigating protein conformation, dynamics and folding with monoclonal antibodies. TIBS **16:**358-362

Gouaux, J.E., O. Braha, M.R. Hobaugh, L. Song, S. Cheley, C. Shustak, and H. Bayley. 1994. Subunit stoichiometry of staphylococcal alpha-hemolysin in crystals and on membranes: a heptameric transmembrane pore. Proc. Natl. Acad. Sci. USA **91:**12828-12831

Gould, K., C.H. Ramirez-Ronda, R.K. Homes and J.P. Sanford. 1975. Adherence of bacteria to heart valves in vitro. J. Clin. Invest. **56:**1364-1370

Greenberg, D.P., J.I. Ward, and A.S. Bayer. 1987. Influence of *Staphylococcus aureus* antibody on experimental endocarditis in rabbits. Infect. Immun. **55:**3030-3034

Greene, C., D. McDevitt, P. Francois, P.E. Vaudaux, D.P. Lew, and T.J. Foster. 1995. Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of the *fnb* genes. Mol. Microbiol. **17:**1143-1152

Greene, C., P.E. Vaudaux, P. Francois, R.A. Proctor, D. McDevitt, and T.J. Foster. 1996. A low-fibronectin-binding mutant of *Staphylococcus aureus* 879R4S has Tn*918* inserted into its single *fnb* gene. Microbiology **142:**2153-2160

Guss, B., M. Uhlen, B. Nilsson, M. Lindberg, J. Sjoquist and J. Sjodahl. 1984. Region X, the cell-wall attachment part of staphylococcal protein A. Eur. J. Biochem. **138:**413-420

Hamill, R.J. 1987. Role of fibronectin in infective endocarditis. Rev. Infect. Dis. **9:**S360-S371

Harlow, E. and D. Lane. 1988. pp.53-138 *In* Antibodies: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Harris, T.O., D. Grossman, and J.W. Kappler. 1993. Lack of complete correlation between emetic and T-cell stimulatory activities of staphylococcal enterotoxins. Infect. Immun. **61:**3175-3183

Hartford, O., P. Francois, P. Vaudaux and T.J. Foster. 1997. The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. Mol. Microbiol. **25:**1065-1076

Hartford, O. Trinity College, Dublin. 1998. Personal communication.

Hawiger, J, S. Timmons, D.D. Strong, B.A. Cottrell, M. Riley and R.F. Doolittle. 1982. Identification of a region of human fibrinogen interacting with staphylococcal clumping factor. Biochemistry, **21:**1407-1413

Hawiger, J., S. Timmons, M. Kloczewiak, D.D. Strong and R.F. Doolittle. 1982. α- and γ-chains of human fibrinogen posess sites reactive with human platelet receptors. Proc. Natl. Acad. Sci U.S.A. **79:**2068-2071

Heinrichs, J.H., M.G. Bayer, and A.L. Cheung. 1996. Characterisation of the *sar* locus and its interaction with *agr* in *Staphylococcus aureus*. J. Bacteriol. **178:**418-423

Heinzel, F.P. 1995. Antibodies. pp36-57 *In* Mandell, G.L., Bennett, J.E., and Dolin, R. (eds.). Principles and practices of infectious diseases. Churchill Livingstone, New York.

Helander, A., H.M.S. Grewal, W. Gaastra, and A-M. Svennerholm. 1997. Detection and characterization of the coli surface antigen 6 of enterotoxigenic *Escherichia coli* strains by using monoclonal antibodies. J. Clin. Microbiol. **35:**867-872

Henrich, B., A. Kitzerow, R-C. Feldmann, H. Schaal, and U. Hadding. 1996. Repetitive elements of the *Mycoplasma hominis* adhesin p50 can be differentiated by monoclonal antibodies. Infect. Immun. **64:**4027-4034

Heraief, E., M.P. Glauser and L.R. Freedman. 1982. Natural history of aortic valve endocarditis in rats. Infect. Immun. **37:**127-131

Herrmann, M., S.J. Suchard, L.A. Boxer, F.A. Waldvogel, and D.P. Lew. 1991. Thrombospondin binds to *Staphylococcus aureus* and promotes staphylococcal adherence to surfaces. Infect. Immun. **59:**279-288

Herrmann, M., Q.J. Lai, R.M. Albrecht, D.F. Mosher, and R.A. Proctor. 1992. Adhesion of *Staphylococcus aureus* to surface-bound platelets: role of fibrinogen/fibrin and platelet integrins. J. Infect. Dis. **167:**312-322

Hienz, S., T. Schennings, A. Heimdahl, and J.I. Flock. 1996. Collagen binding of *Staphylococcus aureus* is a virulence factor in experimental endocarditis. J. Infect. Dis. **174:**83-88

Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F.C. Tenover.. 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J. Antimicrob. Chemother. **40:**135-136

Homonylo McGavin, M., D. Krajewska-Pietrasik, C. Ryden, and M. Hook. 1993. Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity. Infect. Immun. **61:**2479-2485

Hoogenboom, H.R. 1997. Designing and optimizing library selection strategies for generating high affinity antibodies. Trends Biotech. **15:**62-70

Hook, M. .University of Houston, Texas. 1996. Personal communication.

House-Pompeo, K., Y. Xu, D. Joh, P. Speziale, and M. Hook. 1996. Conformational changes in the fibronectin binding MSCRAMMs are induced by ligand binding. J. Biol. Chem. **271:**1379-1384

Hu, D.D., C.F. Barbas, and J.W. Smith. 1996. An allosteric Ca^{2+}-binding site on the β_3 integrins that regulates the dissociation rate for RGD. J. Biol. Chem. **271:**21745-21751

Jameson, B.A., and H. Wolf. 1988. The antigenic index: a novel algorithm for predicting antigenic determinants. CABIOS **4:**181-186

Janzon, L., S. Lofdahl and S. Arvidson. 1990. The role of the δ-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. EMBO J. **9:**1391-1399

Jardetsky, T.S., J.H. Brown, J.C. Gorga, L.J. Stern, R.G. Urban, Y. Chi, C. Stauffacher, J.L. Strominger, and D.C. Wiley. 1994. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. Nature **368:**711-718

Jawetz, E. 1991. Medical Microbiology. Prentice Hall International Inc., London.

Jeljaszewicz, J., L.M. Switalski, and C. Adlam. 1983. Staphylocoagulase and clumping factor, pp 525-577. *In* C.S.F. Easmon and C. Adlam (eds.), Staphylococci and staphylococcal infections, vol. 2. Academic Press Ltd., London

Ji, G., R.C. Beavis, and R.P. Novick. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA **92:**12055-12059

Jonsson, K., C. Signas, H.P. Muller, and M. Lindberg. 1991. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterisation of the second gene. Eur. J. Biochem. **202:**1041-1048

Jonsson, K., D. Mc Devitt, M. Homonylo Mc Gavin, J.M. Patti, and M. Hook. 1996. *Staphylococcus aureus* expresses a major histocompatability complex class II analog. J. Biol. Chem. **270:**1-4

Josefsson, E., K. McCrea, D. Ni Eidhin, D. O'Connell, J. Cox, M. Hook, and T.J. Foster. 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. J. Biol. Chem. **273:**31145-31152

Joseph, G, and E. Pick. 1995. Peptide walking is a novel method for mapping functional domains in proteins. J. Biol. Chem. **270:**29079-29082.

Kaida, S., T. Miyata, Y. Yoshizawa, H. Igarashi, and S. Iwanaga. 1989. Nucleotide and deduced amino acid sequences of the staphylocoagulase gene from *Staphylococcus aureus* strain 213. Nucleic Acids Res. **17:**8871

Kaida, S., T. Miyata, Y. Yoshizawa, S. Kawabata, T. Morita, S. Iwanaga, and H. Igarashi. 1987. Nucleotide sequence of the staphylocoagulase gene: its unique COOH-terminal 8 tandem repeats. J. Biochem. **102:**1177-1186

Kawabata, S., T. Morita, S. Iwanaga, and H. Igarashi. 1985. Enzymatic properties of staphylothrombin, an active molecular complex formed between staphylocoagulase and human prothrombin. J. Biochem. **98:**1603-1614

Kawabata, S., T. Miyata, T. Morita, S. Inagawa, and H. Igarashi. 1986. The amino acid sequence of the procoagulant- and prothrombin-binding domain isolated from staphylocoagulase. J. Biol. Chem. **261:**527-531

Kayser, F.H. 1995. Methicillin and glycopeptide resistance in staphylococci: a new threat? Current Opinion in Infectious diseases **8(suppl. 1):**S7-S11

Kim, J., R.G. Urban, J.L. Strominger, and D.C. Wiley. 1994. Toxic shock syndrome toxin-1 complexed with a class II major histocompatability molecule HLA-DR1. Science **266:**1870-1874

Kloczewiak, M., S. Timmons, and J. Hawiger. 1987. Reactivity of chemically cross-linked fibrinogen and its fragments toward the staphylococcal clumping receptor. Biochemistry **26:**6152-6156

Kochwa, S., R.S. Litwak, R.E. Rosenfield and E.F. Leonard. 1977. Blood elements at foreign surfaces: a biochemical approach to study the adsorption of plasma proteins. Ann. N.Y. Acad. Sci. **283:**37-49

Kolberg, G, and K. Sletten. 1996. Monoclonal antibodies that recognise a common pneumococcal protein with similarities to streptococcal group A surface glyceraldehyde-3-phosphate dehydrogenase. Infect. Immun. **64:**3544-3547

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256:**495-497

Kuypers, J.M. and R.A. Proctor. 1989. Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of *Staphylococcus aureus*. Infect. Immun. **57:**2306-2312

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680-685

Lang, A.B., S.J. Cryz, U. Schurch, M.T. Ganss, and U. Brudurer. 1993. Immunotherapy with human monoclonal antibodies. J. Immunol. **151:**466-472

Lee, J.C., M.J. Betley, C.A. Hopkins, N.E. Perez, and G.B. Pier. 1987. Virulence studies, in mice, of transposon-induced mutants of *Staphylococcus aureus* differing in capsule size. J. Infect. Dis. **156:**741-750

Lee, J.C., N.E. Perez, C.A. Hopkins, and G.B. Pier. 1988. Purified capsular polysaccharide-induced immunity to *Staphylococcus aureus* infection. J. Infect. Dis. **157:**723-730

Lee, J.C., and J. Nemeth. 1995. Antibodies to capsular polysaccharides are not protective against experimental *Staphylococcus aureus* endocarditis. Infect. Immun. **63:**375-380

Lee, J.C., J.S. Park, S.E. Shepherd, V. Carey and A. Fattom. 1997. Protective efficacy of antibodies to the *Staphylococcus aureus* type 5 capsular polysaccharide in a modified model of endocarditis in rats. Infect. Immun. **65:**4146-4151

Lee, J.O., P. Rieu, M.A. Arnaout and R. Liddington. 1995. Crystal structure of the A-domain from the α subunit of integrin CR3 (CD11b/CD18). Cell **80:**631-638

Lee, C.Y., J.J. Schmidt, and A.D. Johnson-Winegar. 1987. Sequence determination of the exfoliative toxin A and toxin B genes from *Staphylococcus aureus*. J. Bacteriol. **169:**3904-3909

Leininger E., P.G. Probst, M.J. Brennan, and J.G. Kenimer. 1993. Inhibition of *Bordetella pertussis* filamentous hemagglutinin-mediated cell adherence with monoclonal antibodies. FEMS Microbiol. Lett. **80:**31-38

Le Vine, H., J-L. Su, and N.E. Sahyoun. 1988. A monoclonal antibody against brain calmodulin-dependant protein kinase type II detects putative conformational changes induced by Ca^{2+}-calmodulin. Biochemistry **27:**6612-6617

Lopes, J.D., M. dos Reis, and R.R. Bretani. 1985. Presence of laminin receptors in *Staphylococcus aureus*. Science **229:**275-277

Lowrance, J.H., L.M. Baddour, and W.A. Simpson. 1990. The role of fibronectin binding in the rat model of experimental endocarditis caused by *Streptococcus sanguis*. J. Clin. Invest. **86:**7-13

Lyon, B.R. and R.A. Skurray. 1987. Antimicrobial resistance pattern of *Staphylococcus aureus*: genetic basis. Microbiol. Rev. **51:**88-134

Mai G.T., J.G. McCormack, W.K. Seow, G.B. Pier, L.A. Jackson, and Y.H. Thong. 1993. Inhibition of adherence of mucoid *Pseudomonas aeruginosa* by alginase, specific monoclonal antibodies and antibiotics. Infect. Immun. **61:**4338-4343

Mamo, W., M. Boden, and J.I. Flock. 1994. Vaccination with *Staphylococcus aureus* fibrinogen binding proteins (FgBPs) reduces colonisation of *Staphylococcus aureus* in a mouse mastitis model. FEMS Immunol. and Med. Microbiol. **10:**47-54

Manzanec, M.B., C.S. Kaetzel, M.E. Lamm, D. Fletcher, and J.G. Nedrud. 1992 Intracellular neutralization of virus by immunoglobulin A antibodies. Proc. Natl. Acad. Sci. **89:**6901-6905

Marx, U., J. Embleton, R. Fischer, F.P. Gruber, U. Hansson, J. Heuer, W.A. de Leeuw, T. Logtenberg, W. Merz, D. Portelle, J-L. Romette, and D.W. Straughan. 1997. Monoclonal antibody production: the report and recommendations of ECVAM workshop. ATLA **25:**121-137

McDevitt, D., P. Vaudaux, and T.J. Foster. 1992. Genetic evidence that bound coagulase of *Staphylococcus aureus* is not coagulase. Infect. Immun. **60:**1514-1523

McDevitt, D., P. Francois, P.Vaudaux, and T.J. Foster. 1994. Molecular characterisation of the fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. Mol. Microbiol. **11:**237-248

McDevitt, D., and T.J. Foster. 1995a. Variation in the size of the repeat region of the fibrinogen receptor (clumping factor) of *Staphylococcus aureus* strains. Microbiology **141:**937-943

McDevitt, D., P. Francois, P. Vaudaux, and T.J. Foster. 1995b. Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus.* Mol. Microbiol. **16:**895-907

McDevitt, D. T. Nanavaty, K. House-Pompeo, E.C. Bell, N. Turner, L. McIntire, T.J. Foster, and M. Hook. 1997. Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. Eur. J. Biochem. **247:**416-424

McDevitt, D. University of Houston, Texas. 1995. Personal communication.

McGavin, M.J., C. Zahradka, K. Rice, and J.E. Scott. 1997. Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. Infect. Immun. **65:**2621-2628

McIntyre, T. Foster and M. Hook. 1997. Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. Eur. J. Biochem. **247:**416-424

Mei, J.-M., F. Nourbakhsh, C.W. Ford, and D.W. Holden. 1997. Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature tagged mutagenesis. Mol. Microbiol. **26:**399-407

Menzies, B.E., and I. Kourteva. 1998. Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis. Infect. Immun. **66:**5994-5998

Mineo, J.R., I.A. Khan, and L.H. Kasper. 1994. A monoclonal antibody that inhibits intracellular replication. Experimental Parasitol. **79:**351-361

Moeck, G.S., P. Tawa, H. Xiang, A.A. Ismail, J.L. Turnbull, and J.W. Coulton. 1996. Ligand-induced conformational change in the ferrichrome-iron receptor of *Escherichia coli* K-12. Mol. Microbiol. **22:**459-471

Moreillon, P., J.M. Entenza, P. Francoili, D. Mc Devitt, T.J. Foster, P. Francois and P. Vaudaux. 1995. Role of *Staphylococcus aureus* coagulase and clumping factor in the pathogenesis of experimental endocarditis. Infect. Immun. **63:**4738-4743

Morfeldt, E., K. Tegmark, and S. Arvidson. (1996). Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Mol. Microbiol. **21:**1227-1237

Mota, G.F.A., Carneiro, C.R.W., Gomes, L., and J.D. Lopes. 1988. Monoclonal antibodies to *Staphylococcus aureus* laminin-binding proteins cross-react with mammalian cells. Infect. Immun. **56:**1580-1584

Much, H. 1908. Uber eine vorstufe des fibrinfer mentes in kulturen von *Staphylokokkus aureus*. Biochem. Z. **14:**143-155

Munro G.H., P. Evans, S. Todryk, P. Buckett, C.G. Kelly, and T. Lehner. 1993. A protein fragment of streptococcal cell surface antigen I/II which prevents adhesion of *Streptococcus mutans*. Infect. Immun. **61:**4590-4598

Munson, S.H., M.T. Tremaine, M.J. Betley, and R.A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. Infect. Immun. **66:**3337-3348

Nanavaty, T. University of Houston, Texas. 1997. Personal communication.

Navarre, W.W., and O. Schneewind. 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol. Microbiol. **14:**115-121

Nelson, L., J.-I. Flock, M. Hook, M. Lindberg, H.P. Muller, and T. Wadstrom. 1992. Adhesins in staphylococcal mastitis as vaccine components. Flemish. Vet. J. **62:**S111-S125

Nemeth, J., and J.C. Lee. 1995. Antibodies to capsular polysaccharides are not protective against experimental *Staphylococcus aureus* endocarditis. Infect. Immun. **63:**375-380

Nesbitt W.E, J.E. Beem, K.P. Leung, S. Stroup, R. Swift, W.P. McArthur, and W.B. Clark. 1996. Inhibition of adherence of *Actinomyces naeslundii* T14V-J1 to saliva-treated hydroxyapatite by a monoclonal antibody to type 1 fimbriae. Oral Microbiol. Immunol. **11**:51-58

Newell, D.G., B.W. McBride, and S.A. Clarke. 1988. pp37-40 *In* Making monoclonals. Public Health Laboratory Service, Colindale, London

Ni Eidhin, D., S. Perkins, P. Francois, P. Vaudaux, M. Hook, and T.J. Foster. 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus.* Mol. Microbiol. **30:**245-257

Nilsson, I.-M., T. Bremnell, C. Ryden, A.L. Cheung, and A. Tarkowski. 1996. Role of the staphylococcal accessory gene regulator (*sar*) in septic arthritis. Infect. Immun. **64:**4438-4433

Nilsson, I.-M., J.C. Lee, T. Bremnell, C. Ryden, and A. Tarkowski. 1997. The role of staphylococcal polysaccharide microcapsule expression in septicemia and septic arthritis. Infect. Immun. **65:**4216-4221

Nilsson, I-M., J.M. Patti, T. Bremell, M. Hook, and A. Tarkowski. 1998. Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus* mediated septic death. J. Clin. Invest. **101:**2640-2649

Novick, R.P. 1967. Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus.* Virology **33:**156-166

O'Callaghan, R.J., M.C. Callegan, J.M. Moreau, L.C. Green, T.J. Foster, O.M. Hartford, L.S. Engel, and J.M. Hill. 1997. Specific roles of alpha-toxin and beta-toxins during staphylococcal corneal infection. Infect. Immun. **65:**1571-1578

O'Connell, D.P., T. Nanavaty, D. McDevitt, S. Gurusiddappa, M. Hook and T.J. Foster. 1998. The fibrinogen-binding MSCRAMM (Clumping Factor) of *Staphylococcus aureus* has a Ca^{2+} dependent inhibitory site. J. Biol. Chem. **273:**6821-6829

O'Connell, D. Trinity College, Dublin. 1998. Personal communication.

O'Toole, P and T.J. Foster. 1986. Molecular cloning and expression of the epidermolytic toxin A gene of *Staphylococcus aureus*. Microb. Pathogen. **1:**583-594

O'Toole, P. and T.J. Foster. 1987. Nucleotide sequence of the epidermolytic toxin A gene of *Staphylococcus aureus*. J. Bacteriol. **169:**3910-3915

Owen, P. 1995. Crossed immunoelectrophoresis in the study of outer membrane antigens, p.237-238. *In* T.K. Korhonen (ed.), Enterobacterial surface antigens: methods for molecular characterisation. Elsevier, Amsterdam, New York, Oxford.

Palma, M., S. Nozohoor, T. Schennings, A. Heimdahl, and J.I. Flock. 1996. Lack of the extracellular 19-kilodalton fibrinogen-binding protein from *Staphylococcus aureus* decreases virulence in experimental wound infection. Infect. Immun. **64:**5284-5289

Park, P.W., D.D. Roberts, L.E. Grosso, W.C. Parks, J. Rosembloom, W.R. Abrams, and R.P. Mecham. 1991. Binding of elastin to *Staphylococcus aureus*. J. Biol. Chem. **266:**23399-23406

Park, P.W., J. Rosembloom, W.R. Abrams,and and R.P. Mecham. 1996. Molecular cloning and expression of the gene for elastin binding protein (EbpS) in *Staphylococcus aureus*. J. Biol. Chem. **271:**15803-15809

Parsonnet, J. 1989. Mediators in the pathogenesis of toxic shock syndrome: overview. Rev. Infect. Dis. **11:**S263-S269

Patel, A.H., P. Nowlan, E.D. Weavers and T.J. Foster. 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. Infect. Immun. **55:**3103-3110

Patti, J.M., H. Jonsson, B. Guss, L.M. Switalski, K. Wiberg, M. Lindberg, and M. Hook. 1992. Molecular characterization and expression of a gene encoding *Staphylococcus aureus* collagen adhesin. J. Biol. Chem. **267:**4766-4772

Patti, J.M., J.O. Boles and M. Hook. 1993. Identification and biochemical characterisation of the ligand-binding domain of the collagen adhesin from *Staphylococcus aureus*. Biochemistry **32:**11428-11435

Patti, J.M., B.L. Allen, M.J. McGavin and M. Hook. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. **48:**585-617

Patti, J.M., T. Bremell, D. Krewjewska-Pietrasik, A. Abdelnour, and A. Tarkowski. 1994. The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. Infect. Immun. **62:**152-161

Patti, J.M., K. House-Pompeo, J.O. Boles, N. Garza, S. Gurusiddappa, and M. Hook. 1995. Critical residues in the ligand-binding site of the *Staphylococcus aureus* collagen-binding adhesin (MSCRAMM). J. Biol. Chem. **270:**12005-12011

Paulsen, I.T., N. Firth, and R.A. Skurray. 1997. Resistance to antimicrobial agents other than β-lactams. pp175-212. *In* K.B. Crossley and G.L. Archer (eds.), The Staphylococci in Human Diseases, Churchill Livingstone, New York.

Paulsson, M., O.D. Liang, F. Ascencio, and T. Wadstrom. 1992. Vitronectin-binding surface proteins of *Staphylococcus aureus*. Zentralbl. Bakteriol. **277:**54-64

Peacock, S.J., T.J. Foster, B.J. Cameron, and A.R. Berendt. 1999. The dominant pathway for *S. aureus* adherence to human endothelial cells in vitro is mediated by fibronectin-binding protein and endothelial cell surface fibronectin. Mol. Microbiol. In press.

Peng, H.L., R.P. Novick, B. Kreiswirth, J. Kornblum and P. Schlievert. 1988. Cloning, characterisation and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **170:**4365-4372

Peterson, C.B., and M.N. Blackburn. 1987. Chromogranin A-like proteins in the secretory granules of a protozoan, *Paramecium tetraurelia*. J. Biol. Chem. **262:**7552-7558

Peterson, P.K., J. Verhoef, L.D. Sabath, and and P.G. Quie. 1977. Effect of Protein A on staphylococcal opsonization. Infect. Immun. **15:**760-764

Peterson, P.K., B.J. Wilkinson, Y. Kim, D. Schmeling, and P.G. Quie. 1978. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. **19:**943-949

Phonimdaeng, P., M. O'Reilly, P.W. O'Toole, and T.J. Foster. 1988. Molecular cloning and expression of the coagulase gene of *Staphylococcus aureus* 8325-4. J. Gen. Microbiol. **134:**75-83

Phonimdaeng, P., M. O'Reilly, P. Nowlan, A.J. Bramley, and T.J. Foster. 1990. The coagulase of *Staphylococcus aureus* 8325-4. Sequence analysis and virulence of site-specific coagulase-deficient mutants. Mol. Microbiol. **4:**393-404

Porath, J., and P. Flodin. 1959. Gel filtration: A method for desalting and group separation. Nature **183:**1657-1659

Prevost, G., P. Couppie, P. Prevost, S. Gayet, P. Petiau, B. Cribier, H. Monteil, and Y. Piemont. 1995. Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. J. Med. Microbiol. **42:**237-245

Projan, S.J., J. Kormblum, and B. Kreiswirth. 1989. Nucleotide sequence: the β-hemolysin gene of *Staphylococcus aureus*. Nucleic Acids Res. **17:**3305

Rampyari, H.R., G Raucci and M. Hook. 1990. Peptide analogs to a fibronectin receptor inhibit attachment of *Staphylococcus aureus* to fibronectin-containing substrates. Infect. Immun. **58:**2593-2598

Raupach, B., and M.A. Schmidt. 1994. Elucidation of linear epitopes of pertussis toxin using overlapping synthetic decapeptides: identification of a human B-cell determinant in the S1 subunit indicative of acute infection. Microbial Pathogenesis **17:**213-226

Rawling, E.G, N.L. Martin, and R.E.W. Hancock. 1995. Epitope mapping of the *Pseudomonas aeruginosa* major outer membrane porin protein OprF. Infect. Immun. **63:**38-42

Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss and R.P. Novick. 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. Mol. Gen. Genet. **202:**58-61

Rozalska, B., and T. Wadstrom. 1993. Protective opsonic activity of antibodies against fibronectin-binding proteins (FnBPs) of *Staphylococcus aureus*. Scand. J. Immunol. **37:**575-580

Rozalska, B., N. Sakata and T. Wadstrom. 1994. *Staphylococcus aureus* fibronectin-binding proteins: identification of antigenic epitopes using polyclonal antibodies. APMIS **102:**112-118

Ryden, C., A.I. Yacoub, I. Maxe, D. Heinegard, A. Oldberg, A. Franzen, A. Ljungh, and K. Rubin. 1989. Specific binding of bone sialoprotein to *Staphylococcus aureus* isolated from patients with osteomyelitis. Eur. J. Biochem. **184:**331-336

Sachse K., H. Pfutzner, M. Heller, and I. Hanel. 1993. Inhibition of *Mycoplasma bovis* cytadherence by a monoclonal antibody and various carbohydrate substances. Vet. Microbiol. **36:**307-316

Sanabria, T.J., J.S. Alpert, R. Goldberg., L.A. Pape, and S.H. Cheeseman. 1990. Increasing frequency of staphylococcal infective endocarditis: experience at a university hospital, 1981 through 1988. Arch. Intern. Med. **150:**1305-1309

Sandre, R.M., and S.D. Shafran. 1996. Infective endocarditis: review of 135 cases over 9 years. Clin. Infect. Dis. **22:**276-286

Sawai, T., K. Tomono, K. Yanagihara, Y. Yamamoto, M. Kaku, Y. Hirakata, H. Koga, T. Tashiro, and S. Kohno. 1997. Role of coagulase in a murine model of hematogenous pulmonary infection induced by intravenous injection of *Staphylococcus aureus* enmeshed in agar beads. Infect. Immun. **65:**466-471

Schennings, T., A. Heimdahl, K. Coster, J.I. Flock. 1993. Immunisation with fibronectin binding protein from *Staphylococcus aureus* protects against experimental endocarditis in rats. Microb. Pathog. **15:**227-236

Schleivert, P.M. 1986. Staphylococcal enterotoxin B and toxic-shock syndrome toxin-1 are significantly associated with nonmenstrual TSS. Lancet **1:**1149

Schleivert, P.M. 1993. Role of superantigens in human disease. J. Infect. Dis. **167:**997-1002

Schneewind, O., D. Mihaylova-Petkov, and P. Model. 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. EMBO J. **12:**4803-4811

Schneewind, O., A. Fowler, and K.F. Faull. 1995. Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. Science **268:**103-106

Shacter, E. 1989. Serum-free media for bulk culture of hybridoma cells and the preparation of monoclonal antibodies. Trends Biotech. **7:**248-253

Shimonkevitz, R., E. Boen, S. Malmstrom, E. Brown, J.M. Hurley, B.L. Kotzin, and M. Matsumura. 1996. Delineation by use of specific monoclonal antibodies of the T-cell receptor and major histocompatablilty complex interaction sites on the superantigen toxic shock syndrome toxin-1. Infect. Immun. **64:**1133-1139

Shuter, J, V.B. Hatcher, and F.D. Lowy. 1996. *Staphylococcus aureus* binding to human nasal mucin, Infect. Immun. **64:**310-318.

Signas, C., G. Raucci, K. Jonsson, P.E. Lindgren, G.M. Anantharamaiah, M. Hook and M. Lindberg. 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: Use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. **86:**699-703

Soell, M., M. Diab, G. Haan-Archipoff, A. Beretz, C. Herbelin, B. Poutrel, and J.-P. Klein. 1995. Capsular polysaccarides types 5 and 8 of *Staphylococcus aureus*: bind to human epithelial (KB) cells, endothelial cells and monocytes and induce release of cytokines. Infect. Immun. **63:**1380-1386

Solomon, B., R. Koppel, D. Kenett, and G. Fleminger. 1988. Localization of a highly immunogenic region of carboxypeptidase A recognized by three different monoclonal antibodies and their use in the detection of subtle conformational alterations in this enzyme region. Biochemistry **28:**1235-1241

Song, L., M.R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, and J.E. Gouaux. 1996. Structure of the staphylococcal α-hemolysin, a heptameric transmembrane pore. Science **274:**1859-1865

Sottile, J., J. Schwarzbauer, J. Selegue, and D.F. Mosher. 1991. Five type I modules of fibronectin form a functional unit that binds to fibroblasts and to *Staphylococcus aureus*. J. Biol. Chem. **266:**12840-12843

Speziale, P., D. Joh, L. Visai, S. Bozzini, K. House-Pompeo, M. Lindberg, and M. Hook. 1996. A monoclonal antibody enhances ligand binding of a fibronectin MSCRAMM (adhesin) from *Streptococcus dysgalactiae*. J. Biol. Chem. **271:**1371-1378

Stahl, S., and M. Uhlen. Bacterial surface display: trends and progress. 1997. Trends Biotech. **15:**185-192

Strong, D.D., M. Moore, B.A. Cottrell, V.L. Bohonus, M. Pontes, B. Evans, M. Riley, and R.F. Doolittle. 1984. Lamprey fibrinogen γ chain: cloning, cDNA sequencing and general characterization. Biochemistry **24:**92-101

Sun, Q., G.M. Smith, C. Zahradka, and M.J. McGavin. 1997. Identification of D motif epitopes in *Staphylococcus aureus* fibronectin-binding protein for the production of antibody inhibitors of fibronectin binding. Infect. Immun. **65:**537-543

Supersac, G., Y. Piemont, G. Prevost, and T.J. Foster. 1997. Assessment of the role of gamma-toxin in experimental opthalmitis using a *hlg*-deficient mutant of *Staphylococcus aureus*. Microb. Pathogen. **24:**241-251

Switalski, L.M. 1976. Isolation and purification of staphylococcal clumping factor, pp413-425. *In* J. Jeljaszewicz (ed.), Staphylococci and staphylococcal infections. Gustav Fischer Verlag, Stuttgart, Germany.

Switalski, L.M., P. Speziale, and M. Hook. 1989. Isolation and characterization of a putative collagen receptor from *Staphylococcus aureus* strain Cowan I. J. Biol. Chem. **264:**21080-21086

Switalski, L.M., J.M. Patti, W. Butcher, A.G. Gristina, P. Speziale, and M. Hook. 1993. A collagen receptor on *Staphylococcus aureus* strains isolated from patients with septic arthritis mediates adhesion to cartilage. Mol. Microbiol. **7:**99-107

Symersky, J. 1997. Structure of the collagen-binding domain from a *Staphylococcus aureus adhesin*. Nat. Struct. Biol. **4:**833-838

Ton-That, H., K.F. Faull, and O. Schneewind. 1997. Anchor structure of staphylococcal surface proteins: a branched peptide that links the carboxy terminus of proteins to the cell wall. J. Biol. Chem. **272:**22285-22292

Toy, P.T.C.Y, L.-W. Lai, T.A. Drake, and M.A. Sande. 1985. Effect of fibronectin on adherence of *Staphylococcus aureus* to fibrin thrombi in vitro. Infect. Immun. **48:**83-86

Tuomanen, E., H. Towbin, G. Rosenfelder, D. Braun, G. Larson, G.C. Hanson, and R. Hill. 1988. Receptor analogs and monoclonal antibodies that inhibit adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells. J. Exp. Med. **168:**267-277

Uhlen, M., B. Guss, B. Nilsson, S. Gatenbeck, C. Philipson, and M. Lindberg. 1984. Complete sequence of the staphylococcal gene encoding protein A. J. Biol. Chem. **259:**1695-1702

Ushioda, H., T. Terayama, S. Sakai, H. Zen-Yoji, M. Nishiwaki, and A. Hidano. 1981. Coagulase typing of Staphylococcus aureus and its application to routine work, p.77-83. *In* J. Jeljaszewicz (ed.), Staphylococci and staphylococcal infections. Gustav Fischer Verlag, Stuttgart, Germany.

Vandenesch, F. J. Kornblum and and R.P. Novick. 1991. A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J. Bacteriol. **173:**6313-6320

Vasfi, M., and K.R. Mittal. 1995. Identification and characterization of outer membrane proteins of *Pasteurella multocida* serotype D by using monoclonal antibodies. J. Clin. Microbiol. **33:**952-957

Vath, G.M., C.A. Earhart, J.V. Rago, M.H. Kim, G.A. Bohach, P.M. Schlievert, and D.H. Ohlendorf. 1997. The structure of the superantigen exfoliative toxin A suggests a novel regulation as a serine protease. Biochemistry, **36:**1559-1566

Vaudaux, P., D. Pittet, A. Haeberli, E. Huggler, U.E. Kydegger, D.P. Lew and F.A. Waldvogel. 1989. Host factors selectively increase staphylococcal adherence on inserted catheters. A role for fibronectin and fibrinogen or fibrin. J. Infect. Dis. **160:**865-875

Vaudaux, P., D. Pittet, H. Haeberli, P.G. Lerch, J.J. Morgenthaler, R.A Proctor, F.A. Waldvogel and D.P. Lew. 1993. Fibronectin is more active than fibrin or fibrinogen in promoting *Staphylococcus aureus* adherence to inserted intravascular devices. J. Infect. Dis. **167:**633-641

Vaudaux, P., V. Monzillo, P. Francois, D.P. Lew, T.J. Foster, and B. Berger-Bachi. 1994. Introduction of the *mec*-element (methicillin resistance) into *Staphylococcus aureus* alters in vitro functional activities of fibrinogen and fibronectin adhesins. Antimicrob. Agents Chemother. **42:**564-570

Vaudaux, P., P. Francois, R.A. Proctor, D. Mc Devitt, T.J. Foster, R.M. Albrecht, D.P. Lew, H. Wabers and S.L Cooper. 1995. Use of adhesion-defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. Infect. Immun. **63:**585-590

Verdrengh, M., and A. Tarkowski. 1997. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. Infect. Immun. **65:**2517-2521

Vincentini, A.P., J.Z. Moraes, J.L. Gesztesi, M.F. Franco, W. De Souza, and J.D. Lopes. 1997. Laminin-binding epitope on gp43 from *Paracoccidioides brasiliensis* is recognised by a monoclonal antibody raised against *Staphylococcus aureus* laminin receptor. J. Med. Vet. Mycol. **35:**37-43

Voet, D and J.G. Voet. 1990. Molecular physiology. *In* Biochemistry, Voet, D and Voet J. (eds.) Wiley, London.

Waldvogel, F. 1995. New insights into septic and toxic diseases produced by *Staphylococcus aureus*. Current Opinion in Infectious diseases **8**(suppl. 1)**:**S3-S6

Wann, E. University of Houston, Texas. 1997. Personal communication.

Wolz, C., D. Mc Devitt, T.J. Foster and A.L. Cheung. 1996. Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64:**3142-3147

Woodin, A.M. 1959. Fractionation of the two components of leukocidin from *Staphylococcus aureus*. Biochem. J. **73:**225-237

Woodin, A.M. 1960 Purification of the two components of leukocidin from *Staphylococcus aureus*. Biochem. J. **75:**158165

Yamada, A., N. Tsukagoshi, and S. Ukada. 1988. Nucleotide sequence and expression in *Escherichia coli* of the gene coding for sphingomyelinase of *Bacillus subtilis*. Eur. J. Biochem. **175:**213-220