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Protein FOG at the Interface between Group G Streptococci and Human Host Defence Lines

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Title and subtitle

Protein FOG at the interface between group G streptococci and human host defence lines

Abstract

Group G streptococci (GGS) may be of four different species and may infect humans and also animals. S. dysgalactiae equisimilis most commonly cause human GGS infections. These bacteria are part of the normal flora, but can cause pharyngitis, erysipelas and impetigo. In the immunocompromised host severe conditions, such as sepsis and necrotising fasciitis, may develop. In this thesis the interactions between GGS and various parts of human defence lines are investigated.

A novel M protein from GGS, denoted FOG, was isolated, recombinantly expressed, and purified. FOG-positive strains survive in human whole blood wheras a FOG-negative strain did not. Addition of soluble FOG, but not protein G, leads to restoration of survival of the FOG-negative strain. Intact protein FOG mediated aggregation of neutrophils in the presence of fibrinogen which disabled these cells in excerting antibacterial activities.

In vitro and in vivo, protein FOG interacts with collagen I, an abundant extracellular matrix protein of human skin. As streptococcal skin infections often preceed invasive disease, the FOG mediated binding to collagen is important and may be the first step of infection. A FOG-positive strain exhibited adhesional advantages compared to a FOG-negative strain.

FOG, like protein G, recruits IgG from human plasma in a non-immune fashion. C1q, initiator of the classical pathway of complement, binds exclusively to the IgG bound via FOG but not to the IgG bound via protein G. IgG opsonisation via FOG but not via protein G led to an O2- production by neutrophils.

FOG is released from the bacterial surface into the growth medium of bacterial early stationary growth phase and also by neutrophil elastase. Like M1, an M protein of GAS, FOG binds to monocytes and triggers secretion of the chemokines MIG (CXCL9) and IL-8 (CXCL-8). GGS are less susceptible than GAS, to antimicrobial effects of MIG in physiological NaCl concentrations corresponding to that of sweat and plasma but not of saliva, and also to these NaCl concentrations alone.

Key words: FOG, group G streptococci, M protein, virulence, adhesion, fibrinogen, phagocytosis, collagen, protein G, C1q, protein interactions

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To Mom and Dad

Hur kan Du vara så säker på Ditt svar?
Hur kan Du vara så iskall, ren och klar
när det finns platser i Vintergatan kvar
som vi aldrig får se?
J. Berg

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV).

   I   *Protein FOG- a streptococcal inhibitor of neutrophil function*
        Johansson H.M., Mörgelin M., Frick I-M.
        Microbiology 2004, 150;4211-4221.

   II   *Streptococcal protein FOG: a novel matrix adhesin interacting with collagen I in vivo*
        Nitsche D.P.*, Johansson H.M *, Frick I-M., Mörgelin M.
        J Biol Chem. 2006, 20;281(3):1670-9.

   III   *Differential Effects of Group G Streptococcal IgG-Binding Proteins on Innate Immune Defense*
        Nitsche D.P., Johansson H.M., Sastalla I., Reismann S., Frick I.-M., and Chhatwal G. S.
        Manuscript

   IV   *Promoting survival of a commensal: A and G streptococcal variation in induction of, and susceptibility to the antibacterial chemokine MIG*
        Johansson H.M., Egesten A., Frick I.-M.
        Manuscript

* indicates these authors as equally contributing

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Abbreviations

α2M α 2 macroglobulin
aa amino acid residues
AMPs antimicrobial peptides
ARF acute rheumatic fever
C1q complement factor 1 q
CR complement receptor
E Enterococcus
ECM extracellular matrix
Fab fragment, antigen binding
Fc fragment, chrystallizable or constant
FcR Fc receptor
FOG Fibrinogen binding protein of G streptococci
G(**+**), or (**-**) Gram positive, or negative
GAGs glycosaminoglycans
GAS group A streptococcus
GBS Group B streptococcus
GCS group C streptococcus
GGS group G streptococcus
IFNγ interferon γ
Ig immunoglobulin
IL-8 interleukin 8
MAC membrane attack complex
MBL mannose binding lectin
Mga multigene regulator of GAS
MSCRAMMs microbial surface components recognizing adhesive matrix molecules
NADPH nicotinamide adenine dinucleotide phosphate
OF opacity factor
RHD rheumatic heart disease
ROS reactive oxygen species
Sfb1streptococcal fibronectin binding protein 1
Spe streptococcal pyrogenic exotoxin
TLR Toll-like receptor

Introduction

Everyday humans encounter a diversity of microbes. Most are harmless to us, some are helpful, but some cause infections and disease. A pathogen is defined as a microbe with the ability to cause disease. Bacteria, fungi, viruses and parasites are collectively referred to as microbes. An array of microbes which live in symbiosis with the host and do not cause disease under normal conditions, is termed the *normal flora*. However, for various reasons e.g. immunological status of the host, stress or antibiotic treatment, the balance of symbiosis may be altered resulting in the unlimited growth, spread and survival of otherwise harmless microbes. If and when these microbes are able to cause disease they are called *opportunists*. Microbial factors which promote the ability to cause disease are referred to as *virulence factors*. This thesis deals with a virulence factor, called protein FOG, at the interface between group G streptococci and the human host. In this thesis, I will describe the human host defence lines (foremost the innate immunity) and how some microbes benefit from interactions therewith. Then, the bacterium will be presented along with molecular links to streptococcal pathologic states. Finally, the current investigations will be addressed.

The Lines of Defence

The human defence system can be divided into two parts, the innate and the adaptive. The innate defence is comprised of a number of barriers, ions, compounds and proteins with direct or indirect antimicrobial activities. The innate immunity is rapid in response and is equally potent upon re-encounters with pathogens. It was first believed that species evolutionarily distant from humans, lacked an overall immune defence due to their lack of adaptive immune system. It became evident that this was not the case and that e.g. invertebrates shared traits of antimicrobial recognition and killing with higher vertebrates. Since this discovery, innate immunity has been the object of intense research. The adaptive immune system requires priming and takes longer time to mobilize. The B and T lymphocytes which act as controlers of immunoglobulin (Ig; antibody) production and recognition of self and non-self, are examples of adaptive immunity players. Actions like antigen presentation and signalling between cells, are convergence points between innate and adaptive immunity. The two systems partly overlap to ensure a sufficient immunoresponse towards pathogens.

The five cardinal symptoms of inflammation are redness, warmth, pain, swelling and the possible loss of function in the affected limb or area. The symptoms are partly due to an increase of vascular permeability caused by inflammatory mediators released from immunological cells which have reacted to tissue damage, invading microbes or an otherwise disturbed homeostasis. Acute inflammation is the peak of an immunoresponse, which starts with the mounting i.e. production and release of signalling cytokines and the recruitment of phagocytic cells. Inflammation is most oftenly resolved in an active process which requires production and release of mediators of wound healing, cell migration and cell proliferation.

Barriers

The utmost outer line of human defence is comprised of physical barriers. The skin and mucosal linings of the oral cavity, airways, gut and urogental tracts are efficient in blocking entry paths of pathogens. The barriers are built up by a number of resident cell types. Although being resident, keratinocytes, macrophages, dendritic cells, and epithelial cells act as sentinels and exert defensive actions by production of antimicrobial peptides (AMPs), cytokines and antigen presentation. The fluids

covering the barriers contain antimicrobial substances (1). These include lactoferrin which sequesters iron which is necessary for bacterial respiration and lysozyme which cleaves the N-acetyl bonds of the peptidoglycan present in the cell wall of G**+** bacteria. The pH and ionicity of body fluids act in a bacteriostatic fashion. The composition of **sweat** involves ions such as Na^+, Cl^-, K^+ and Mg^{2+} and a degree of acidity (pH 4,5-6) (2) but with variations due to nutritional and immunologic status (3, 4). **Saliva** is secreted into the oral cavity and contains Na^+, Cl^-, and K^+, along with albumin, lysozyme and cathelicidins (5). The **airways** are lined with a fine mucosal layer, covered by a film of fluid. Lactoferrin and lysozyme together with other constitutively expressed antimicrobial agents manage to keep the lower airways sterile and the upper airways from being colonised by pathogens (6).

Antimicrobial peptides

AMPs have recently been recognised as an important part of the innate immune defence. Such peptides are present at all levels of phyologeny, from invertebrates to humans (7). They are included in the first line of defence towards microbes and are present on skin, mucosal surfaces and in various body fluids. It may also be that AMPs have evolved to control the bacterial counts of the normal flora. Generally AMPs are 15-45 amino acid residues (aa) in length and carry a positive net charge which allows the interaction with the negatively charged bacterial membrane. Actions of AMPs may be mediated by i) insertion into the microbial membrane followed by pore formation which causes lysis, ii) membrane insertion through a carpet formation of a large amount of AMP causes lysis, iii) intracellular targeting. The exact mode of lysis is not altogether clear. PR-39, an AMP from pig intestine, inhibits bacterial DNA and protein synthesis, indicating a possibility of non-membrane orientated modes of AMP action (8).

In humans, the two major families of AMPs are the cathelicidins and the defensins (7). A cathelicidin is characterized by a highly conserved N-terminus and a microbicidal activity located in the C-terminal part of the peptide. The defensins are of the α, β or θ classes. Structurally, the three main groups of AMPs are i) α-helical without cystein residues, ii) peptides with three disulphide bonds, or iii) peptides rich in a certain aa such as histidine or arginine. In case of AMPs, the structure is of great importance for efficient killing (9, 10) . An increased ionic strength is believed to alter the structure and thereby inhibiting the microbicidal effect.

The most well studied human cathelicidin, LL-37, is derived from a precursor called hCAP-18 and belongs to the α-helical group (11-13). It is a linear peptide, processed from hCAP-18 by tissue specific proteases to generate active peptides (14, 15). The α- and β-defensins which belong to the disulphide bond group, and LL-37 are fundamental to the human innate immune system (7). Morbus Kostmann is a severe recessive disorder which results in neutropenia and a lack of neutrophil and salivary LL-37. These patients suffer from recurrent infections and the state is lethal if untreated. Cleavage of LL-37 by bacterial proteases favors bacterial survival (16). Also, these proteases release host dermatan sulphate which inactivates α-defensins (17).

Active forms of AMPs are secreted but may also be generated through proteolytic cleavage of proteins with unknown and sometimes unrelated "day-time jobs" (18, 19). Through *in silico* searches, a multitude of proteins have been found to contain antimicrobial motifs. The presence and *in vivo* relevance of such are difficult to estimate. Many AMPs are inactivated by increased ionicity and presence of plasma. AMPs are produced by several cell types e.g. epithelial cells, fibroblasts, keratinocytes, monocytes, and neutrophils (7). Most AMPs are active against a broad

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spectrum of microbes and synergistic activity between AMPs of different classes have been demonstrated (20). Their antimicrobial effects and rapid action limits the risk of development of bacterial resistance mechansims. This motivates AMPs as candidates for novel treatment strategies. As host cells are protected by the relative neutral surface charge of their plasma membrane, the risk of side effects is reduced.

Chemokines

Inflammatory chemokines are small (70-130 aa) chemotactic cytokines, secreted by cells upon encounter with trauma, pathogens or inflammatory mediators (21). Chemokines act locally and systemic levels do not necessarily reflect the inflammatory process. They are categorized dependent on the intramolecular pattern of conserved cystein residues (22). The four groups are the XC, CC, CXC and CX_3C chemokines. These may be ELR positive (+) or negative (-), dependent on the presence of an ELR aa motif. Chemokines as ligands in signalling are denoted "L" e.g. CXC<u>L</u> and the chemokine receptors are denoted "R" e.g. CC<u>R</u>.

Interferon γ (INFγ) is a proinflammatory cytokine. The chemokines MIG, IP-10, and ITAC (monokine induced by IFNγ or CXCL9, IFNγ-inducible protein 10 or CXCL10 and IFN-inducible T cell α chemoattractant or CXCL11, respectively) are under positive genetic regulation of IFNγ. MIG recruits natural killer cells and T cells, through CXCR3 present on these cells. The expression of MIG is elevated in psoriatic skin (23) and in synovial fluid of patients suffering from rheumatoid arthritis (24, 25). MIG also functions as an AMP (26) and its role in fighting group A streptococcal (GAS) infection in a pharyngitis model has been demonstrated (Egesten *et al In preparation*). A synthetic peptide derived from the C-terminal region of the MIG molecule, showed antibacterial activity comparable to that of the intact protein. This indicated that this α-helical part is responsible for the bactericidal activity. MIG is ELR-, does not attract neutrophils and is expressed in the later phase of wound healing (27).

In contrast to MIG, interleukin (IL)-8 (CXCL8) is ELR+, negatively regulated by IFNγ and exerts chemotactic and activating effects on neutrophils by interaction with the receptors CXCR1 and CXCR2 (21). IL-8 is reported to have a positive effect on wound healing (28, 29) and neutrophil migration (30). Possibly, this could be due to an antimicrobial motif which was recently recognized within the IL-8 sequence (31). In addition, IL-8 engagement of chondrocytes may alter expression of remodeling enzymes in cartilage matrix (21). A multitude of studies of IL-8 and infectious disease concern various streptococcal species, foremost pneumococci (*Streptococcus pneumoniae*) and group B streptococci (GBS, *S. agalactiae*) (32-34) but little is reported for GGS. Inactivation of IL-8 is achieved by proteolytic cleavage by novel GAS enzymes (35, 36).

Immunoglobulins

Following phagocytosis and destruction of encountered microbes, the components of the microbe, the so called *antigens* are presented on the surface of the ingesting cell. An interaction between the antigen presenting phagocyte and circulating B lymphocytes results in B cell maturation into plasma cells. Each plasma cell will produce and secrete Ig's, which is designed to target the particular antigen that was presented to its B cell. The mission of Ig's is to recognise and mediate destruction of microbes or foreign matter through interaction with Fc-receptors on phagocytic cells or by initiating the classical pathway of the complement system. Ig's are also present in the plasma membrane of B cells.

Each Ig consists of two types of polypeptide chains. These are the heavy and light chains and are linked together by covalent and non-covalent bonds. The classes of Ig's are named by their heavy chain isotype and are IgA, IgE, IgD, IgM and IgG.The IgG molecule has a variable antigen binding region (Fab) and a constant region (Fc) which interacts with cells of the immune system e.g. neutrophils and macrophages. Fab and Fc are joined together by a hinge region. IgG and IgA can be further divided into subclasses 1-4. The majority (60%) of the IgG in circulation is IgG1, followed by IgG2 (30%), IgG3 (7%) and then IgG4 (3%). IgG3 differs from the other subclasses in its structure as it has a longer hinge region. Interestingly, the hinge region of IgG is prone to cleavage by the GAS proteases SpeB and IdeS (37, 38).

Physiologically, IgG-mediated effects involve binding of antigen through the Fab region, but bacterial and endogenous proteins with affinity for the Fc region have also been identified. A detailed description of non-immune binding of IgG is depicted in Figure 1 and a description of Fc-binding proteins can be found below. Aggregated IgG and also IgM can bind complement factor 1q (C1q) and thereby initiate the classical pathway of complement (described below). The interaction site for C1q on the IgG molecule has been localised to the Cγ2 domain of IgG (39).
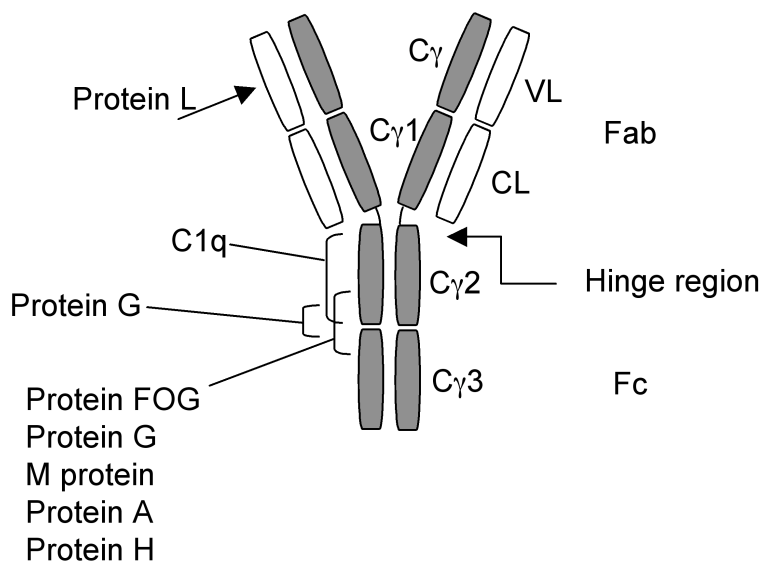

Figure 1. Depiction of an IgG molecule. Heavy chains are in grey wheras light chains are in white. Regions are noted to the right and binding sites for bacterial surface proteins and C1q are indicated to the left.

The Complement system

The complement system is a defence system found in blood. It is comprised of more than 30 soluble and cell bound proteins. The system is tightly regulated and requires activation to exert its actions. The innate and adaptive immune systems converge in the complement system which controls recognition of non-self, chemotaxis, phagocytosis, and bacterial lysis. The complement system can be activated in three different ways: the classical, the alternative, and the lectin pathway. Complement deposition is an example of *opsonisation*, which is marking a foreign surface for destruction by phagocytosis or lysis.

The classical pathway

The classical pathway of complement is initiated by the binding of C1q (in complex with C1r and C1s, the so called C1 complex) to pentameric IgM or aggregated IgG (40). Cleavage of C1s by the autocatalytic C1r renders the C1 complex enzymatically active and ready to cleave C4 and C2 in a Ca^{2+} dependent manner. This results in the formation of the classical C3 convertase (C4b2a) which cleaves C3, a protein circulating in plasma, to C3a and C3b. The latter is a potent opsonin which then

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covalently attaches to the antigen. The former is an anaphylatoxin with recently discovered antimicrobial potential (19). The classical pathway is dependent on presence of antibodies, Ca^{2+}, and Mg^{2+}.

The alternative pathway
The alternative pathway of complement activation is primarily antibody independent and provides a rapid amplification of the classical pathway, resulting in cascade deposition of C3b on foreign surfaces such as prosthetic implants (40). The alternative pathway occurs via spontaneous activation of plasma C3 to $C3(H_2O)$. Factor B binds to $C3(H_2O)$ in a Mg^{2+} dependent manner and becomes available for cleavage by factor D. Cleavage of the complexed factor B occurs, releasing C3Ba, but leaving part of factor B (Bb) in place. The Bb part of this complex is enzymatically active and cleaves C3 to C3a and C3b. C3b may associate with Bb and form C3bBb, the C3 convertase of the alternative pathway. Properdin stabilizes this complex and prolongs its half-life. The C3 convertase cleaves C3 to C3a and C3b, of which the latter can join together with Bb to form yet another C3bBb, an amplification loop.

Lectin pathway
Mannose binding lectin (MBL) is a collagenous pattern recognition molecule with a mission to recognize and bind bacterial carbohydrates (40). Upon engagement, a complex of MBL and MBL-associated serine proteases is formed. This complex activates C4 and the downstream activation is identical to that of the classical pathway.

Convergent pathway of complement and regulation
The two different forms of C3 convertase, bind to and cleave C3 as described and in the process also yeild the C5 convertases C4b2a3b and C3bBb3b. These bind to and cleave C5, the initiator of the formation of the membrane attack complex (MAC). MAC is a complex of complement factors C5-C9 which forms pores resulting in lysis of target cells. The thickness of the cell wall of G**+** is thought to protect from MAC mediated lysis and hence G**-** bacteria are primarily affected. Despite this, certain GAS strains express a protein called <u>s</u>treptococcal <u>i</u>nhibitor of <u>c</u>omplement (*sic*), which interferes with the formation of MAC (41).

The complement system is a powerful system which, if uncontrolled, could cause considerable damage to the host. For example, C3b does not discriminate between self and foreign surfaces. To ensure protection from the harmful effects, fluid phase and membrane associated proteins tightly control complement activation (40). This is achieved by mechanisms that i) increase decay of active complement complexes (e.g. factor H) or ii) support and enhance the effects of factor I. Factor I is a serine protease which cleaves C3a and b and thereby limits their actions. The only known positive regulator of complement is properdin which has been described above.

C1q
C1q is a 460 kDa innate immune defence molecule with the structural appearance of a bunch of tulips (42). The collagenous region of C1q comprises the N-terminal stem and the globular heads, which interact with IgG, are depicted as the C-terminal bulbs. The molecule is comprised of 18 polypeptide chains: 6 A, 6 B and 6 C chains.

Besides initiating the classical pathway of complement, C1q is implicated in various immunomodulating actions (for references see (42)). Among these are that C1q is chemotactic for neutrophils and eosinophiles and an interaction between its collagen-like region and neutrophils, induces O_2^- production. C1q may act as an opsonin by direct binding to microbes and also triggers the release of proinflammatory cytokines like IL-8 and IL-6 from endothelial cells. C1q induces apoptosis in fibroblasts and is

reported to have antiproliferative effects on cell lines. Interactions with a broad range of ligands suggests that C1q recognizes a pattern of charged aa or groups of aa within the target protein. This has made the search for cell surface receptors difficult (43). On neutrophils the C1q receptor is unidentified but lipid rafts have been suggested to play a role in the O_2^- production mediated by C1q-binding (44). Genetic deficiency of C1q is associated with systemic lupus erytematosus and a disabled clearance of antigen-antibody complexes, which results in glomerulonephritis (42). C1q is a member of the collectin family, where other members include MBL, and surfactant types A and D. Prevention of activation of the classical pathway by binding of decorin and biglycan to C1q was recently reported (45). These two ligands are abundant extracellular matrix (ECM) proteoglycans which may regulate complement activation at the tissue level.

The Arg[114] of the globular heads of the B chains of C1q is crucial for IgG binding and Arg[129], Arg[163] and His[117] make important contributions, as shown by mutational analysis (46). In human IgG, the region of residues 270-333 of Cγ2 are indicated as important for interaction with C1q (47-49). In **paper III**, we examine the C1q-binding to IgG bound to the G streptococcal surface via proteins FOG and G, respectively. We found that C1q binds to IgG that was bound via protein FOG, whereas IgG bound to the bacterial surface via protein G was inaccessible for C1q interaction.

Phagocytes and phagocytosis

Phagocytosis is the act of ingestion of microbes, apoptotic cells or debris and results in the elimination of unwanted particles. The particle to be ingested is first bound to surface receptors of the phagocytic cell and is sequentially taken up into an intracellular compartment termed *phagosome*. Phagocytic cells include neutrophils, monocytes/macrophages, NK-cells, and dendritic cells. The degree of eosinophil phagocytosis may vary with state of activation by proinflammatory cytokines.

Monocytes

Like neutrophils, monocytes are of myeloid lineage and develop in the bone marrow. Monocytes reach their target tissue via the circulation and mature into dendritic cells or site specific macrophages, i.e. Langerhan cells of the skin, Kupffer cells of the liver, podocytes of the kidneys, and osteoclasts of the bone and cartilage. Despite the relative small number of monocytes in circulation (only 10% of the total number of mononuclear cells) they comprise a strong link between the innate and adaptive immune system. They do so by raising an amplificational cascade of inflammatory mediators to alert, attract and activate endothelial cells, neutrophils and T-cells subsequent to pathogen recognition (50).

Neutrophilic granulocytes

Neutrophilic granulocytes, also referred to as polymorphic neutrophils, are the major phagocytic cells of the bloodstream. Upon activation, neutrophils are able to leave the circulation through a process called diapedes and migrate towards a gradient of chemotactic molecules to reach the site of an infection. High levels of chemokines cause the release of granule content from neutrophils and enhance the antimicrobial mission of cells of the immune system. Chemotactic mediators can be of both host (e.g. C3a and C5a) and microbial (e.g. fMLP, lipoteichoic acid, peptidoglycan and other structural components) origin. The life span of a neutrophil is short, 2-3 days in circulation, but they are of utmost importance in fighting pathogens as indicated by the increased risk of infections in patients with neutropenia.

Despite a great capacity of phagocytosis, neutrophils and macrophages may suffer from exaggerated activation or so called *frustrated phagocytosis*. This may occur

upon attempt to ingest large particles and also on experimental surfaces. Surface adherence and activation of neutrophils may lead to a cellular depletion of lytic factors resulting in an impaired microbicidal function (51). One study showed that the capacity of mouse macrophages to phagocytise was dependent on the availability of plasma membrane rather than the availability of free receptors (52). The same study indicated 15 μm as the size limit of unopsonised particles.

Receptors of phagocytosis

Phagocytosis and the subsequent events are triggered by engagement with specific surface receptors. These are the Fc receptors (FcRs), complement receptors (CRs) and pattern recognition receptors, respectively.

FcγRs recognize the Fc region of IgG. In this context, both neutrophils and macrophages express FcγRI and FcγIIRa wheras neutrophils also express FcγRIIIb (53, 54). Both neutrophils and macrophages express CR1, CR3 and CR4 which lead to or directly mediate phagocytosis by recognition of C3b or C3bi (54). Pattern recognition receptors recognize conserved motifs or patterns which are specific to microbes. The Toll-like receptors (TLRs) belong to this group. TLR1, 2, and 4-9 are expressed on neutrophils (55). Monocytic TLRs include TLR1, 2, and 4 (1). Recent research in the streptococcal field indicates that CR3 (CD11b/CD18 or Mac-1) is responsible for binding and subsequent phagocytosis of M5 GAS by neutrophils (56, 57).

Killing mechanisms

Traditionally, the killing of microbes by the human neutrophils and macrophages may be divided into oxygen dependent and independent strategies.

Oxidative burst

The oxygen dependent mechanism of microbe killing by phagocytic cells involves the formation of an active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Its mission is to produce microbicidal reactive oxygen species (ROS) through a process called *respiratory* or *oxidative burst* (58). The NADPH oxidase is composed of four cytosolic proteins (p40^{phox}, p47^{phox}, p67^{phox} and rac2) and one membrane bound (flavocytochrome b, a heterodimer of gp91^{phox} and p22^{phox}). Upon activation, the cytosolic proteins co-localize in the outer plasma membrane or in the membrane of the phagosome, which contains the ingested microbe. Once assembled, the NADPH oxidase reduces extracellular or intraphagosomal oxygen molecules (O_2^-) to superoxide ions (O_2^-) which may convert to hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\bullet$) and hypochlorous acid (HCl^-) (59). The importance of oxidative burst in innate immunity is exemplified in chronic granulomatous disease (CGD). Patients suffering from CGD, caused by inherited mutations in the proteins of the NADPH oxidase (60), are subject to recurrent infections (61).

Recently, focus shifted from the microbicidal actions of ROS and instead, towards the importance of the lytic contents of neutrophil granules ((62) and references therein). The suggested role of the NADPH oxidase is to depolarize the phagosomal membrane and by influx of potassium ions cause liberation of elastase and cathepsin G from the proteoglycan matrix, to which these enzymes are bound in an inactive state. This was supported by the fact that mice genetically deficient in neutrophil elastase and cathepsin G, were unable to kill infecting pathogens although the levels of ROS were normal.

| |
|---|
| **1. Secretory vesicles** |
| albumin |
| membrane components |
| CR3 |
| flavoprotein B |
| **2. Gelatinase granules** |
| gelatinase |
| lactoferrin |
| **3. Specific granules** |
| lactoferrin |
| transcobalamin II |
| gelatinase-associated lipocalin |
| membrane components |
| lysozyme |
| flavocytochrom B |
| **4. Azurophilic granules** |
| MPO |
| cathepsin G |
| elastase |
| proteinase 3 |
| lysozyme |
| defensins |
| Bactericidal Permeability Increasing protein (BPI) |
| Proteoglycans |
| HBP |
| **5. Lysosomes** |
| acid hydrolases |
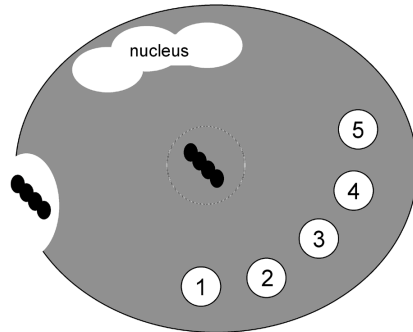
Figure 2. The content of neutrophilic granules are listed and numbers indicate order of fusion with the membrane of the phagosome or the neutrophil plasma membrane.

Neutrophil granule actions

The oxygen independent mechanisms of neutrophil killing involve release of granules substances which aid in microbial killing. The azurophilic, specific, gelatinase granules and secretory vesicles and their respective contents is displayed in Figure 2. In the process of neutrophil activation, the secretory vesicles are the first to exert their action. By fusing with the plasma membrane, the content of the secretory vesicles prime the cell into a CR3 expressing neutrophil (63). This transforms it to a migratory and responsive cell. The gelatinase granules then fuse with the plasma membrane. Their contents allow degradation of collagen and hence a free tissue passage for a migrating neutrophil (64). The specific granules then fuse and due to the rich contents of their membranes in flavocytochrome b, these granules are NADPH oxidase recruiting. Degranulation of the azurophilic granules, releases lytic and bactericidal proteins into the phagosome and to the extracellular environment through fusion with the phagosomal or plasma membrane, respectively. Finally, the lysosomes fuse with the phagosome and subject its contents to acid hydrolases.

Neutrophil elastase, a potent serine protease stored in the azurophilic granules, can directly kill G- bacteria (65) and has been shown to release surface components from A and G streptococci (66, 67). Cathepsin G and proteinase 3 are also potent enzymes exhibiting direct microbicidal effects (68, 69). Neutrophil proteases cleave several proteins implicated in wound healing, cell signalling, and antimicrobial activities (70, 71). Processing enables modulation of e.g. IL-8 to a decreased or an increased potency or may alter target specificity.

Group G streptococci

In 1933, Rebecca Lancefield grouped streptococci based on group specific carbohydrate antigens (72). The β-hemolytical streptococci, displaying a clearing zone surrounding the colonies when grown on blood agar, are of groups A, B, C, D, F and G (73). Although rare, groups E, P, U and V may also be included.

Group G streptococci (GGS) expressing the G specific carbohydrate antigen include 4 different species: *Streptococcus (S) dysgalactiae equisimilis*, *S. equi zooepidemicus*, *S. canis* and *S. anginosus* (73). Taken together, GGS can act as both commensals and pathogens in animals and humans. However, strains of *S. dysgalactiae equisimilis* are mainly associated with human infections and may carry antigens A, C, G or L, with G and C being the most common. *S. equi zooepidemicus* is regarded as a commensal in horses, and the cause of bovine mastitis, but has also caused outbreaks of severe human disease. The G antigen is also carried by strains of *S. canis*. As the name indicates these are mainly found in dogs, but have caused disease in humans. However, the characteristics of isolates found in dogs and humans respectively, have not been thoroughly investigated. The *S. anginosus* group is heterogenic with regard to pattern of hemolysis, and may be subgrouped into *S. anginosus*, *S. constellatus* and *S. intermedius*. In the *anginosus* group, F is the most common antigen followed by C, A and most seldomly G. Virulence of this group has been questioned. The *S. anginosus* subgroup, which can also lack specific antigen, may be found in the urogenital and gastrointestinal tracts of humans. *S. constellatus* is a respiratory tract finding, whereas *S. intermedius* has been found in brain and liver abcesses.

The species mentioned above are distinct from *S. dysgalactiae dysgalactiae,* and *S. equi equi* which cause bovine mastitis and equine respiratory disease, respectively (73). The former is not β hemolytical and the latter carries the C carbohydrate antigen. Neither have been associated with human infections unlike the more well studied GAS (*S. pyogenes*) which have humans as exclusive host.

The nomenclature of GGS has been confused as types, displaying markers formerly distinct for the different groups, have been isolated and caused a clouded classification (74-76). Although such cases are rare, this demonstrates a difficulty in determining the true cause of an infection, and may also show the need for novel identification strategies, rather than depending on carbohydrate antigen grouping. To date, clinical isolates of β-hemolytical G**+** cocci displaying streptococcal group G specific carbohydrate showing resistance to bacitracin, capability of deamination of arginine, hydrolysis of esculin, and production of acid in trehalose and ribose broth, are termed *S. dysgalactiae equisimilis* (73). For clarity, the strains in focus in the current investigation are clinical isolates from human infections caused by GGS and will be referred to as such.

Clinical spectrum and epidemiology

S. dysgalactiae equisimilis is by far the most commonly isolated GGS in human infection although GGS in general have been regarded as commensals. Reports covering the last decades demonstrate an increased severity of clinical manifestations caused by GGS. An overview of recent case reports is given in Table 1. The clinical spectrum of disease caused by GGS is similar to that of GAS. Most commonly, GGS cause superficial dermal lesions such as impetigo and erysipelas. Pharyngitis and non-symptomatic carriage on human skin, and mucosal surfaces in the throat, gastrointestinal and vaginal tracts are also common. However, invasive disease such as bacteremia, cellulitis and necrotising fasciitis can cause life threatening conditions in the immuno-compromised or elderly host (77). Chronic wounds, ulcers and prosthetic implants may also host GGS (78-80). In developed countries, an association is seen between GGS infections and increased age and underlying malignancies i.e. diabetes mellitus, cardiovascular conditions (77, 81, 82) or drug abuse (83). Lymphatic disorders also appear to be a risk factor. Case reports include patients whom in the past, have undergone radiation treatment for malignancies of the pelvic region (84, 85). A common site of GGS infection is the thigh, buttocks and hip.

In tropical areas, streptococcal skin infections are more common than in temperate areas where pharyngitis is the leading manifestation of GAS (86). GAS are commonly found in skin sores in the aboriginal population, contributing to the morbidity of this group (87). Also, asymptomatic pharyngeal carriage of GGS and group C streptococci (GCS) within this group is suggested to play a role in the development of acute rheumatic fever (ARF), a well known streptococcal sequel disease (88). Other post streptococcal diseases known from GAS, and also described for GGS are glomerulonephritis and post-streptococcal arthritis (79, 89-91).

Genomics of GGS

The GGS are a heterogeneous population of organisms and with high degree of genetic plasticity. The phenotypical heterogeneity has been explained by an ongoing transfer of genes from other bacterial species (92). Proteins associated with virulence, namely C5a peptidase and M protein, were first found in GAS and later identified in human, but not necessarily animal isolates of GCS and GGS (93, 94). Sequencing of housekeeping genes from large numbers of isolates show genetic relationships between GCS and GGS and also horisontal transfers of genes from GAS to GGS and GCS (95, 96). Analysis show the transfers as recent events. The shift of genetic material from GGS to GAS or vice versa are suggested to be due to phage preference or may coincide with the domestication of animals.

Superantigens are well known secreted molecules in GAS pathogenesis but their role in GGS pathogenesis has only recently come into focus. Streptococcal pyrogenic exotoxins (Spe's) A, C and G, J, M, *ssa* and *smeZ* have been reported in strains of GGS and GCS (96-99). Interestingly, despite a 87% similarity to SpeG of GAS, the SpeG found in GGS was functionally inert in inducing a mitogenic response in lymphocytes (99). Reports suggest various Spe:s as phage encoded (100). Of note, in GAS experimental transfer of a gene encoding an antiphagocytic M protein, into a new genetic background within the same species, did not result in an antiphagocytic strain (101). This may represent a requirement for additional genetic regions for adequate expression. This may also represent a limiting factor in gene transfer between GGS and GAS. Despite efforts SpeB, an important enzyme and virulence factor of GAS, has not been reported for GGS (102).

Table 1. A number of case reports and summarizing studies of GGS clinical cases.
1.cancer/malignancy 2. diabetes 3. cardiovascular related disease *n.n. not noted

Clinical manifestation	Source of isolation	Underlying malignancy	Age	Location, Year	Ref.
Septic chock (n=2)	*n.n.	none	n.n.	Belgium 1996	(103)
Recurrent cellulitis	blood culture	1	67	U.S Florida 1998	(85)
Meningitis	blood culture	3	83	Belgium 2000	(104)
Necrotizing fasciitis	*n.n.	2	52	U.S. Detroit 2002	(105)
Purulent pericarditis	pericardial fluid	1	52	Korea, 2002	(106)
Toxic shock syndrome	blood culture, hip bullae	1	73	U.S Florida 2003	(84)
Osteomyelitis	Trucut biopsy femur head	none	71	Hong Kong, 2003	(107)

Summarizing reports

Number of cases, % of β-h-strep examined	Source of isolation	Underlying malignancy	Age	Location, Year	Ref.
(n=26) 20%	Bacteremia, (blood)	1, 2, 3, ulcers, alcoholism, i.v. drug use	32-96	UK, 2002	(77)
Screening study (n=69) 21%	respiratory tract, soft tissues, reproductive tract	*n.n.		India, 2004	(108)
Invasive (n=16) *n.n.	blood, abcess-, surgical-, joint fluid	1, 2, 3 cirrhosis, renal disease, *tinea pedis*, osteoarthiritis	43-90	Japan, 2003	(109)
Retrospective (n=94) 19%	cellulitis, blood	1, 2, 3	2-92	Israel, 1989-2000	(81)

The M protein

The M protein confers the trait of phagocytosis resistance and is traditionally regarded as a major streptococcal virulence factor. It was first discovered in GAS and is encoded by a gene termed *emm*, which is chromosomally located downstream from the *mga* (multigene regulator of GAS) regulon. Also situated on the *mga* regulon is *mrp*, *enn* (additional M or M-like proteins; (110)) *sic* (41), C5a peptidase and opacity factor (OF). The composition of individual mga controlled proteins differ dependent on presence (+) or absence (-) of OF. In strains of GGS and GCS, a *mga*-like regulon has been found (111) and termed *mgc*. However, *mgc* showed a significant divergence both in the up- and downstream regions compared to *mga*. (112) An *enbloc* transfer of the regulon from GAS to GGS, followed by deletion and rearrangements has been suggested. In *S. dysgalactiae* of bovine mastitis, demA and demB, two M like proteins, were found downstream from dmgA and dmgB which were found to be similar to the mga regulon of GAS (113). Research in this area indicates the genomes of GCS and GGS as highly mosaic and exhibiting a level of plasticity.

Immunization of extracts from streptococcal cultures gave rise to type specific opsonic antibodies, which were used for serotyping. Traditionally, this was the way of M typing individual strains. Today, a molecular based typing system is used of foremost GAS, but also groups G, C and L (http://www.cdc.gov/ncidod/biotech/strep/emmtypes.htm, (73)). To date there are more than 120 serotypes known for GAS and approximately 40 for the groups C, G and L. Two isolates are regarded as sharing the same *emm* type if they are > 95% identical over their 5' end 160 nucleotides (which includes part of the signal peptide) using defined PCR primers. Lancefields grouping of serotypes 7, 16, 20, and 21 were mistaken for GAS but were in fact, GGS and GCS.

The M proteins of GAS are classified as type I or type II based on the C-terminal aa sequence (114). Only strains expressing type II M proteins produce OF. M proteins vary in size between 25 and 80 kDa and are anchored to the cell wall through an LPXTG motif typical for surface proteins of G**+** bacteria (115, 116). The highly conserved C-terminal region is typically comprised of 3 C-repeats, a mid-orientated S region followed by B-repeats and in some cases, A repeats. The N-terminal half is considered semivariable and the absolute N-terminus is classified as hypervariable. The M protein dimerizes into a coiled-coil structure with temperature dependent stability (117-119). The stability of the coiled-coil structure also increases upon ligand binding. The helical content is greater in the C-terminal part and contributes to stability. Furthermore, the M protein is accessible for ligand interaction even after heating to 80°C. The development of a vaccin against GAS infections is focused on the M protein although the M type diversity poises a limitation of vaccin efficacy.
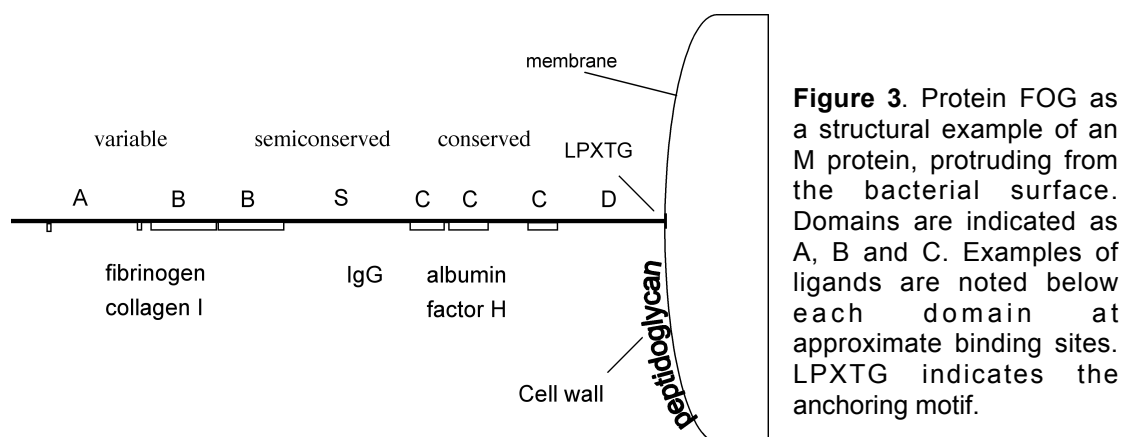

Figure 3. Protein FOG as a structural example of an M protein, protruding from the bacterial surface. Domains are indicated as A, B and C. Examples of ligands are noted below each domain at approximate binding sites. LPXTG indicates the anchoring motif.

M protein as a virulence factor

As early as 1964, Kantor showed that M protein could interact with the human plasma protein fibrinogen (120). Survival of M protein expressing strains was seen in plasma but not serum, demonstrating the role of fibrinogen. Whitnack and Beachey related surface bound fibrinogen to a hindered deposition of complement on the surface of M positive strains (121). The binding of fibrinogen was first located to the N-terminal part of the molecule (122) and then mapped to the B repeats of the molecule (123). Different M types bind to different parts of fibrinogen, implying a convergent evolution and an importance for this binding. Multiple studies confirm the role of fibrinogen in phagocytosis resistance (123-125). Sandin *et al* (126) showed that binding of antibodies directed towards the fibrinogen binding region of the M protein, is masked by fibrinogen and that only antibodies directed towards the N-terminal hypervariable region opsonize the bacteria.

Also, M proteins limit streptococcal binding to neutrophils (57), but promote adhesion (127, 128) and invasion (129) of resident celltypes. A large number of tested M proteins bound to the glycosaminoglycans (GAGs) of host proteins of the ECM and enabled GAS adherence to human fibroblasts and epithelial cells (130). In addition, interactions between GAS M1 protein and fibrinogen form complexes that induce vascular leakage which contributes to the severity of sepsis (67). Interactions with other plasma proteins such as Ig's, albumin, plasminogen, kininogen and complement factors are shown by a multitude of studies (129, 131-133)

M proteins of GGS

Early studies implied the presence of M proteins in GGS (134), and that these shared properties with the M proteins of GAS (93, 135-137). In 1992, Collins *et al* reported the first complete gene sequence for an M protein from a human GGS strain (138). The deduced protein shared structural homologies with the M proteins of GAS, and it was classified as being of type I. In 1995, it was summarized that 100% of human GGS strains (38 strains and 2 for comparison; (138, 139)) examined until 1995, were OF- and the M proteins were of type I (94). A PCR based typing method for GGS revealed homologies between the GGS *emm*-like genes (called *emmL*) and GAS types *emm57* and *emm12* and it was confirmed that strains of human but not animal origin, expressed M protein. Owing to the type I classification of the GGS M proteins it appears likely that GGS *emm* genes have evolved from a common ancestor. It is also plausible that the homologies are a result of horisontal gene transfer rather than convergent evolution. By restriction analysis some studies show that virulent GGS could be limited to a few strains which have spread (140). Others have shown that sharing the same emm type not necessarily proves the strains as clonal (81).

A recent study showed that a large proportion (155/214, 72%) of the tested GGS and GCS strains from human pharyngeal infections in Spain were ≥95% identical of *emmL* types previously entered in GenBank (141). In the large number of investigated strains, 23% (50/214) were non-typeable and almost half of those were from the *S. anginosus* subgroup. This study resulted in the report of nine novel *emmL* types. The most commonly isolated *emm* types of GCS/GGS in this study (*emmC839*, *fcrV*, *emm28D*, *emmC1139* and *mlc36*) did not overlap with the most prevalent GAS *emm* sequences (*emm1*, *3*, *12*, and *9*).

The majority of reported GGS M proteins from human infections have been examined for emm type and binding properties *in vitro*. In *emm* typing studies, partial gene sequences are yeilded but not expressed as proteins and hence not directly examined for roles in virulence. Protein FOG is investigated for interactions with neutrophils, monocytes, and skin (**this thesis**). Protein FOG which shares the typical structural appearance of a GAS M protein is depicted in Figure 3.

Investigators have identified M or M-like proteins in *S. dysgalactiae equisimilis*, *S. equi zooepidemicus, S. dysgalactiae dysgalactiae*, and *S. equi equi* (142-144). The M-like protein SzPSe of *S. zooepidemicus* is α-helical and fibrillar but does not contain the classical repeated domains found in M proteins of GGS and GAS (143).

Mediators of bacterial aggregation

The ability of bacteria to aggregate may mediate a level of protection against host defence. Phagocytosis may be impeded as larger bacterial aggregates are difficult to ingest. A defined sequence (aa 150-168) in the M-like protein H of GAS mediates aggregation and adherence to host cells (145). This may facilitate colonisation. Other microbes such as *Bordetella pertussis*, *Staphylococcus aureus*, enterotoxigenic *Escherichia coli*, *Mycobacterium tuberculosis* and *bovis* display similar aggregational properties (146-149). Studies of cell surface hydrophobicity of streptococcal groups A, B, C, D and G revealed a higher degree in M-exressing GAS than in the other groups (150). In this study, GBS were relatively hydrophilic wheras the GCS and GGS aggregated to the same level as M negative GAS when examined in increasing ionicity. It is however unclear whether the GGS and GCS in this study were M protein expressing or not. It was also demonstrated that binding of fragment D of fibrinogen to M protein of GAS, specifically reduced cell surface hydrophobicity (151). This is interesting as the physiochemical properties of bacteria may influnce interactions with host molecules, cells, or tissues.

Protein G and other bacterial Fc-binding proteins

Bacteria are able to bind IgG in a non-immune fashion i.e. via Fc and not via the Fab region. This binding is mediated by surface proteins which are of great experimental value in experimental purification of IgG from complex solutions. Fc-binding among G**+** bacteria has been classified as being of types I-V, based on animal species and IgG subclass specificity (152). Examples of such proteins are: protein A of *Staph. aureus* (Fc type I binding), streptococcal M and M-like proteins such as protein H (type II binding), and protein G of C and G streptococci (type III binding). Additionally, protein L of *Finegoldia magna* (formerly *Peptostreptococcus magnus*) binds the light chains of IgG which enables these bacteria to bind to all Ig isotypes (153). Non-immune binding of IgG is summarized in Figure 1. The role of Fc-binding has been suggested to act as an environmental sensor or as a case of molecular mimicry to avoid host recognition (154).

Recently, it was found that protein A provokes an inflammatory response in human airway epithelia by interaction with the tumor necrosis factor receptor 1 (155). In the case of protein G, a great deal of information is available, but a conclusive role in virulence or otherwise is still lacking. Protein G (65 kDa) was discovered in C and G streptococci by two independent groups (156, 157). It binds IgGFc with high affinity, broad species and subclass specificity and differs from protein A in that it also binds IgG3. The binding site within protein G is mapped to the C-terminal part of the C domains (158). There are no significant sequence homologies between the IgG-binding regions of proteins A and G. The proteins differ in pH optimum for IgG-binding which suggests the need for different environments to adopt a correct structure (159). This also supports the IgG-binding of these proteins as a case of convergent evolution. Protein G binds IgGs of many animal species, wheras GAS M proteins primarily bind human IgG. As GGS can infect animals as well as humans, and GAS exclusively infect humans, a role in virulence for protein G has been suggested. Protein FOG, an M protein of GGS (**paper I**) however binds animal IgG with broad specificity (**paper III**). There were no differences in protein G expression when strains from severe disease were compared with those of milder infections (159). Intact protein G has mitotic activity on lymphocytes, but not resulting from IgG-binding (160).

Proteins A and G show a weak affinity for the Fab region of IgG (156, 161). More interestingly the respective binding of proteins A, G and H to IgG could all be blocked by a synthetic peptide based on the Fc-binding region of protein G (158). This

indicates that they all bind to the same or closely located region in IgG, the interface of the Cγ2 and Cγ3 domains (162). Berge et al (163) examined complement activation after adding Fc-binding proteins A, G, M and H to serum. Activation was seen in all cases except for M protein. In the fluid phase, the classical pathway was activated and IgG and C1q shown to be responsible for protein H mediated conversion of C3. In the solid phase, when IgG was immobilized on beads or the bacterial surface, protein H inhibited complement activation. However, when protein G bound to IgG which was immobilised on beads the level of complement activation increased. This is in contrast to our findings in **paper III** where we show that C1q only binds to IgG presented by protein FOG but is unable to bind to IgG bound to the bacterial surface via protein G. In the perspective of C1q opsonisation, our results demonstrate protein G as an anti-opsonin.

Protein G also binds human serum albumin, the protease inhibitor α2 macroglobulin (α2M) and kininogen (164). Protein PAB, with homology to protein G, mediates albumin binding, a feature of strains of *F. magna* causing localized suppurative infections but not of commensal strains (165). In GAS, the protein G related surface protein GRAB recruits SpeB to its surface via bound α2M, which enables the degradation of AMPs (166, 167). Whether protein G shares these traits has not been studied.

Proteins exhibiting type III IgG binding were found in a group C *S. zooepidemicus* (168), *S. dysgalactiae* from bovine mastitis (169), and *S. dysgalactiae* (170). These proteins are called ZAG, MAG and MIG and vary in number of IgG binding domains and share partial sequence homology with protein G. Additionally they bind α2M, and except for MIG, also human serum albumin. It was found that MIG conferred phagocytosis resistance by bovine neutrophils and this was related to binding of α2M rather than IgG (171).

Other virulence factors reported for GGS

Fom inside to outside, the G+ bacterium is comprised of cytosol, a single membrane and the cell wall. The cell wall is composed of a macromolecule of peptidoglycan with associated structures such as lipotechoic acids and carbohydrates. Located in the cell wall are LPXTG anchored proteins out of which many are implicated in virulence. One such protein is the **C5a peptidase** which degrades C5a and was first found in GAS and GBS (172, 173). GGS strains of human but not animal infections were reported to harbor a C5a peptidase which was similar to that of GAS (174). Most GGS harbor one or both of the **streptolysin types O** and **S** (73). These secreted lysins are the cause of the hemolysis zone surrounding each colony forming unit when bacteria are grown on blood agar plates. In strains isolated from cases of severe GGS infection researchers linked streptolysin S expression to virulence of both GGS and GAS in a mouse model (175). Hemolysin deficient GGS strains may be overlooked as cause of pharyngitis (176) and since GGS have been suggested to cause post streptococcal sequelae, this is an important finding. **Streptokinase** is a secreted activator of plasminogen and implicated in streptococcal dissemination. There is also evidence for a role of streptokinase in poststreptococcal glomerulonephritis (177)

Bacterial interactions with extracellular matrix

The interstitium between the cells of the body, is a network of proteins which constitutes the ECM. The ECM provides a structural and supportive framework, used by migrating and maturing cells. Certain ECM proteins exist exclusively in the interstitium, whereas others also circulate in blood. In addition, ECM is involved in wound healing and tissue remodelling (178) and also provides a dynamic interaction partner for growth factors, cytokines (30) and antimicrobial agents (21).

Bacteria employ the ECM to facilitate adhesion through proteins known as adhesins or microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (179). A microbial surface protein which binds with considerable specificity and affinity to an ECM component may be regarded as an MSCRAMM. That ECM ligands vary in tissue distribution may explain the tissue trophism noted for infections cause by microbes expressing MSCRAMMs or adhesins.

GAGs are composed of repeating units of amino sugars and uronic acids. These form linear sulphated polysaccharide chains which are usually attached to a protein core. Together these form a proteoglycan. GAGs are expressed on cell surfaces and in the ECM and may be classified based on their disaccharide composition. Examples of GAGs are dermatan sulphate, chondroitin sulphate, heparan sulphate and heparin. GAGs function as receptors in M protein-mediated adhesion of GAS (130).

Fibrinogen

Fibrinogen is a 340 kDa glycoprotein synthesized in the liver (180). The symmetrical molecule, circulating in plasma, is made up of 3 pairs (i.e. 6 chains) of peptide chains termed α, β and γ. Thrombin, generated by the coagulation cascade, cleaves fibrinogen and releases fibrinopeptides A and B. This exposes polymerization sites in the E domain which leads to incorporation of multiple cleaved fibrinogen molecules, resulting in the formation of a fibrin clot. Fibrinogen can also engage platelets which aggregate and stimulate clot formation. Polymorphisms in the fibrinogen gene and proteolytic processing by neutrophils, suggest a possible myriad of variants of fibrinogen within the same individual. Fibrinogen and its cleavage products have implications in cell migration during wound healing (181, 182), neutrophil activation (183) and can act as a regulator of cytokine production (184).
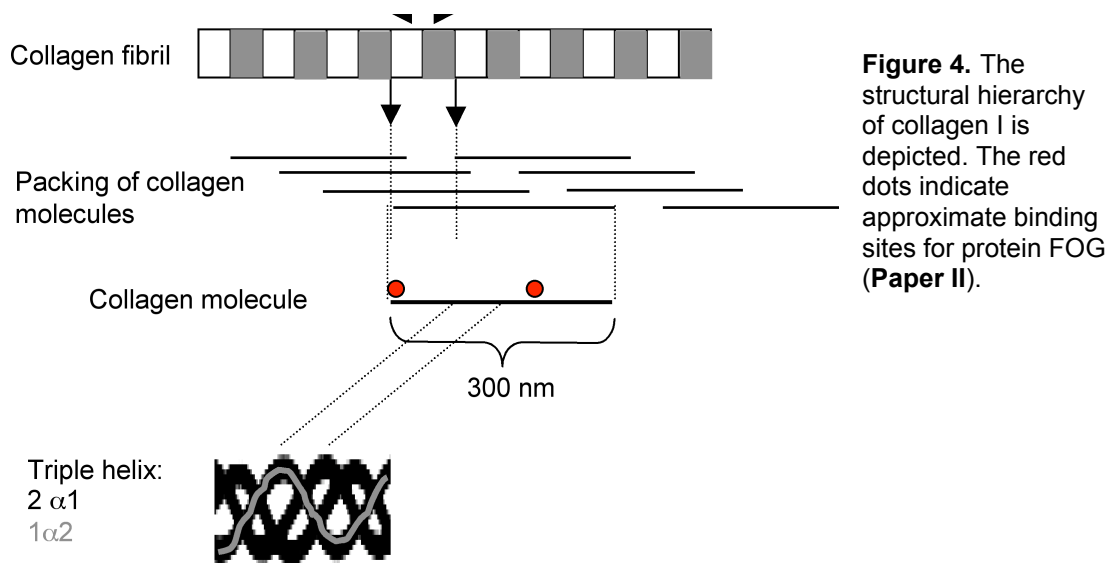
Streptococcal proteins have been found to aggregate fibrinogen in a non-enzymatic fashion. Aggregates examined by electron microscopy show an appearance distinct from that of thrombin induced network formation. FbsA, a fibrinogen binding surface protein of GBS, causes an extraordinary degree of aggregation (185). Similar complex formation between an M protein of GAS and fibrinogen has been reported (67). A *Staph. aureus* protein termed clumping factor, interacts with fibrinogen (179). Interestingly, GAS M proteins along with *Candida albicans*, and *Aspergillus fumigatus* bind to the D domain of fibrinogen.

Fibronectin

A large body of evidence supports the role of fibronectin in bacterial adhesion. Fibronectin is a dimeric 440 kDa glycoprotein, found in the ECM and in plasma. In GAS, Sfb1/protein F (186, 187) is a well defined adhesin with implications in host cell adhesion and internalisation (188, 189). In human disease associated GGS, one study showed that a surface protein termed group G streptococcal fibronectin binding protein (GfbA) through interaction with fibronectin, mediated GGS adherence to human skin fibroblasts (190). A partial sequence homology between GfbA and Sfb1 of GAS was identified.

Collagen

There are 26 forms of collagens, with varying distribution in ECM of the human body. They are divided into fibrillar and non-fibrillar forms and referred to by roman numerals indicating the chronology of discovery. Collagens I, II and III are the major fibrous collagens. Collagen I (Figure 4), a heterotrimer composed of two identical $\alpha1$ chains and one non-identical $\alpha2$ chain, is important in cell-matrix adhesion and cell differentiation. The 3 α chains form a helix where every third aa is a glycine, followed by a proline. Collagen I is found in skin and connective tissue and totals 90% of the collagen content of the body. Collagen II is extensively expressed in joints and collagen III, found in tendons, skin and vascular structures. Collagen IV is the structural framework of basal membranes.

Figure 4. The structural hierarchy of collagen I is depicted. The red dots indicate approximate binding sites for protein FOG (**Paper II**).

Various microbes express surface proteins with the ability to bind to collagen. A selection of surface proteins from G+ bacteria is displayed in table 2. A collagen binding protein of GAS, denoted CPA, was found in 30% of investigated strains (191). Its role in adhesion to collagen I has been determined and the binding was mapped to the A region in CPA. Interestingly, the B region of CPA shows significant homology to the B repeated region of CNA, a collagen I binding protein from *Staph. aureus* (192). The role of the B region of these proteins, is unknown. Expresssion of CNA is linked to adhesional advantages of *Staph aureus* and also induced septic arthritis in a mouse model. Also, the affinity to collagen correlated well to the severity of infection. Lannergård *et al* (193) found a CNA homolog (CNE) in *S. equi equi*. The highest homology between the two proteins was found in the N-terminal A region, responsible for collagen binding.

Furthermore, Dinkla *et al* have reported a fibronectin-dependent recruitment of collagen I and IV via Sfb1 in GAS (194). This bridging mechanism protected the strains from neutrophil phagocytosis. A direct binding of collagen I was reported in only 10% of the investigated strains. Besides Sfb1, the capsule of investigated GAS, and protein M3, have been described as collagen binding (195). Complementing adhesional advantages, collagen binding is suggested to link streptococcal infection to ARF. Experiments, where immunization of mice with M3 protein led to production of antibodies towards collagen type IV, are in support of this. In **paper II,** we demonstrate a mode of GGS adherance to human and murine skin through a direct binding between protein FOG and collagen I.

Table 2. A selection of collagen binding proteins in G**+** bacteria.
n.i. not investigated, n.n. not noted, E. *Enterococcus*

Microbial protein	Role	Bacteria	Collagen type	Reference
n.i.	n.i.	A, B, C, D, G streptococci	I	(179)
57 kDa	adhesin	GAS	I-IV	(196)
CPA	adhesin, internalisation	GAS	I	(191)
n.i.	n.i.	GAS	IV	(197)
FOG	adhesion	GGS	I	**Paper II**
CNE	adhesion	*S. equi equi*	I	(193)
FNZ	adhesion, signalling	*S. equi zooepidemicus*	I	(198)
n.i.	n.i.	*S. dysgalactiae*	I	(199)
cnm-encoded	adhesin	*S. mutans*	I	(200)
antigen I / II	n.i.	*S. mutans*	I	(201)
16 kDa	adhesion	*S. mutans*	I	(202)
Cna	MSCRAMM	*Staph. aureus*	II	(203)
Ace	MSCRAMM	*E. faecalis*	I	(204)
SalA, salB		*E. faecalis*	I	(205)
Acm	MSCRAMM	*E. faecium*	I	(206)
Cbpa	adhesion	*Arcanobacterium pyogenes*	I	(207)

Molecular links to streptococcal sequelae

ARF, RHD

The link between streptococcal infection and ARF and rheumatic heart disease (RHD) has long been under investigation. In the western world, the incidence is less than 10 in 100 000 individuals (for references see (208)). However, in less developed communities the incidence reaches 500 in 100 000. In rising economies, ARF incidence is falling. ARF is caused by cummulative damage to the heart and valves. Recurrent infections are thought to cause ARF, but only one untreated infection may be enough to cause disease. Onset of ARF is usually just prior to adolescence and as affected individuals reach the age of 25-30, the state has progressed to RHD.

In general, ARF is regarded as a GAS associated disease (208). Classically, certain GAS mucoid strains have been categorized as rheumatogenic, in particular M18. Surprisingly, these strains are rare in areas where ARF disease is common. Also, strains causing pharyngitis but not skin infections have been classified as rheumatogenic. This appears true in temperate areas where streptococcal infection on a whole is low. However, in tropical areas and in communities where hygiene conditions are low and crowded living is a problem, streptococcal strain plentitude may overturn this correlation. Although ARF and RHD are common in certain indiginous populations i.e. the Maori of New Zealand and the aboriginies of central and northern Australia, no host specific predisposing factor has been conclusively established. In contrast, in the latter group pharyngeal carriage of GAS was low, whereas GCS and GGS were more prevalent. These have been suggested to take part in priming individuals who later on will acquire GAS infection followed by ARF. Antibodies raised against bacterial surface proteins cross react with tissues of the joints and heart. The M protein has been favored in this context, due to generation of antibodies that cross react with cardiac myosin and laminin, possibly through coiled-coil structure homologies. High antibody titers against group A specific carbohydrate have also been seen in patients suffering from ARF.

Glomerulonephritis

Glomerulonephritis is a renal condition involving the glomeruli, which may follow streptococcal infection. GAS are commonly isolated in these cases, but GGS and GCS have been reported as causative agents (89, 91). Children are most oftenly affected and the condition is often self-limiting. The effects of streptococcal binding to ECM molecules, and the presence of streptokinase has been examined in this context (177).

Present investigation

Paper I Isolation, expression and characterization of protein FOG

It has previously been shown that M proteins present on GGS confer antiphagocytosis in human whole blood, to expressing strains (93, 135, 136). The binding of fibrinogen to M proteins circumvent deposition of complement factors (209). The role of protein G in survival in human whole blood had however not been investigated. By comparing the survival of 10 GGS strains, we found that all except one strain (G148) survived and multiplied in human whole blood. Knowing that these strains express protein G on their surface, we decided to investigate what contributed to phagocytosis resistance in the other strains. Strain G41 was selected for further investigation. Suspecting the presence of an M or M-like protein, a chemically cleaved surface protein of G41 which interacted with fibrinogen was sequenced and recombinantly expressed in *E. coli*. The protein, denoted FOG, showed structural homologies to other M proteins of GAS and GGS, with 2 short A-repeats located N-terminally, 2 unique B-repeats, an S-region, 3 C-repeats followed by a C-terminal D-region with an LPXTG motif anchoring the protein to the bacterial cell wall (see figure 3). Recombinant expression of the protein and fragments thereof were used to map the interaction sites with the plasma proteins IgG, fibrinogen, albumin and factor H of complement. The binding of factor H occurred both to protein FOG as well as protein G, i.e. binding was evident to both strains G41 and G148. Hence, we could rule out a major input of factor H binding when investigating bacterial survival in whole blood. In addition, complement deposition by the alternative pathway did not differ between G41 and G148. FOG mediates bacterial aggregation which is reported to increase virulence.

When adding soluble FOG to whole blood, macroscopic aggregates were seen on the wall of the test tube. Presence of fibrinogen and neutrophils in the aggregates was demonstrated. Protein FOG could also aggregate purified human neutrophils in the apparent absence of fibrinogen. However, it was shown that the purified neutrophils carry immobilised fibrinogen on their surface, possibly as a result of activation during purification. The presence of this fibrinogen might explain the FOG induced aggregation. Incubation of soluble protein FOG and the FOG-negative strain G148 in whole blood, resulted in bacterial survival. Interestingly, a recombinant fragment of FOG with retained fibrinogen-binding did not mediate survival of G148. Using a longer FOG fragment resulted in a maintained but not increased number of surviving bacteria. For a complete rescue of G148, the full length protein FOG was required. In contrast, addition of soluble protein G or the unrelated protein PAB of *F. magna* did not lead to bacterial survival or aggregation. We could also show that a FOG-expressing strain is able to rescue a non-expressing strain, pointing to a possibility of problems occurring with polymicrobial infection.

Binding of fibrinogen to M proteins has long been implicated in streptococcal survival. We could conclude that despite a retained ability to bind fibrinogen, the shorter recombinant fragments of protein FOG did not mediate beneficial consequences for the bacteria. Such consequences involve neutrophil adhesion, phagocytosis or the subsequent killing. The results emphasize the importance of an intact FOG molecule as it is presented on the bacterial surface for full bacterial survival.

Taken together a new GGS M protein denoted FOG (accession no. AY600861), with unique B-repeats was isolated, purified and recombinantly expressed. Its role in bacterial survival was mediated by aggregation of neutrophils in the presence of fibrinogen. In spite of this, the ability to bind fibrinogen does not guarantee survival in whole blood.

Paper II Protein FOG is an adhesin which binds to collagen I of human and murine dermis

The skin is regarded as an important site of entry for GGS infections. Cases of severe disease has often been preceded by infections of superficial location e.g. the skin. We show that G41 bacteria adhered in greater numbers to human skin sections and biopsies than did G148. A search for a possible binding partner in human skin was conducted by probing extracts of human skin, separated by SDS-PAGE, with radiolabelled recombinant FOG. Protein bands interacting with the probe, were analysed by mass spectrometry and identified as collagen I. The interaction site on FOG for collagen I was investigated by use of recombinant FOG fragments by surface plasmon resonance analysis and electron microscopy. The binding site was located in the N-terminal half of protein FOG. On collagen I, the interaction site was found on the very tip of the collagen triple helix, as well as 235 nm distal from this tip. As the collagen was extracted with pepsin, we could not distinguish between the N- and C-terminal end of the molecule. However, no additional binding sites on collagen were observed and this allowed a relative definition of the binding site.

In contrast to GAS, the investigated GGS strains could bind collagen directly. It has been shown that the binding of collagen by GAS in a large number of strains was mediated by bridging of fibronectin (194). Through competetive binding assays we show that collagen binding is mediated by FOG and not protein G. We successfully blocked bacterial adherence to human skin sections with soluble collagen I, indicating that this molecule is a likely interaction partner in human skin. We could demonstrate *in vivo* relevance of this interaction, by showing a greater number of G41 rather than G148 bacteria adhering to mouse dermis and human fibroblasts stimulated to ECM deposition. In summary, we show that protein FOG, through interaction with collagen I, acts as an adhesin to human skin and may initiate a GGS infection.

Paper III FOG promotes opsonisation by C1q whereas protein G does not

Complement deposition is an essential part of the innate host defence. The effects of the classical, alternative and lectin pathways are manipulated by streptococci to promote survival (210). We show that C1q, the instigator of the classical pathway, can bind exclusively to FOG-bound IgG but not to IgG bound by protein G. As FOG can detach from the bacterial surface as well as by neutrophil elastase (**paper IV**) this gives the bacterium a possibility to evade C1q depostion. Furthermore, protein G acts as an antiopsonin, by sterically blocking C1q docking to IgG. The relevance of the two populations of IgG on the bacterial surface was examined by analysis of neutrophil O_2^- production. Indeed, G148 preadsorbed with IgG yielded less O_2^- production from neutrophils than did G41. Mapping of the IgG binding site in FOG was investigated and located to the C terminal part of the S-region. Protein FOG was found to bind to animal IgGs with a broad species specificity. IgG-opsonisation through FOG but not protein G led to an O_2^- production by neutrophils.

Paper IV GGS are tolerant to NaCl concentrations of sweat and plasma and less affected by MIG in these NaCl concentrations than GAS.

It has been shown that M protein of GAS can be cleaved from the bacterial surface by endogenous or host proteases (67, 211). Such a release of protein G has been described (66). We demostrate that also FOG is released from the bacterial surface by neutrophil elastase and also found in medium from bacterial stationary phase of growth. The impact of the released FOG was compared to that of M1 in binding to monocytes. Both bacterial proteins bound to monocytes and evoked a secretion of IL-8 and MIG. The MIG response was more rapid and reached higher levels when cells were stimulated with protein M1. This finding was confirmed by real-time PCR.

The levels were also higher for heatkilled whole AP1 bacteria of GAS compared to G41 and G148 of GGS.

When investigating the sucseptibility of GGS and GAS to the bactericidal effects of MIG, we saw no difference between the two species. However, with increasing ionic strength, GGS were proven less susceptible to MIG. Also, GGS were more tolerant to NaCl concentrations corresponding to that of sweat and plasma. This could be a previously overlooked passive trait of survival in these organisms. In speculation, a difference in bacterial net surface charge between GGS and GAS could explain the difference in susceptibility to antimicrobial peptides.

Conclusions

- o Protein FOG, a novel M protein of GGS, exerts effects on neutrophils which renders them incapable of bacterial killing.
- o Protein G is not responsible for bacterial survival in human whole blood.
- o Expression of protein FOG relates adhesional advantages as it binds to collagen I of human and murine skin.
- o In concentrations of NaCl corresponding to that of sweat and plasma, GGS are less susceptible to the antibacterial effects of MIG than GAS although both species and their respective M proteins trigger the release of CXC chemokines from monocytes.
- o GGS are better survivors than GAS in concentrations of NaCl corresponding to that of sweat and plasma but not of saliva.
- o Protein G counteracts IgG mediated oxidative burst in neutrophils whereas FOG bound IgG is still capable of evoking this response.
- o The C1q binding site in IgG is blocked for interaction, when IgG is bound to the bacterial surface via protein G, but not via protein FOG.

Populärvetenskaplig sammanfattning på svenska

Mikrober och immunförsvaret

I våra dagliga liv möter vi mikroorganismer av olika slag, vilka i stigande storleksgrad kan delas in i virus, bakterier, svampar och parasiter. En del av dessa orsakar sjukdom och kallas därför patogener. Virulensfaktorer kallas de mikrobiella faktorer eller egenskaper som är nödvändiga för att framkalla sjukdom. Sådana faktorer kan mediera tex vidhäftning till värdorganismens celler för vidare kolonisering, möjlighet att undgå upptäckt av värdens immunförsvar eller att underlätta spridning i vävnad eller blod. Som motpart till mikrobernas arsenal av virulensfaktorer har värdorganismer som t.ex. människan utvecklat ett immunförsvar. Detta kan indelas i två olika delar: det medfödda och det förvärvade. Det medfödda immunförsvaret består bl a av fysiska barriärer som hud och slemhinnor, ämnen som är direkt eller indirekt mikrobdödande, och fagocyterande celler vilka har till uppgift att äta upp och eliminera inkräktare. Det medfödda immunförsvaret kännetecknas av snabb respons och att det lika lätt känner igen patogener varje gång de påträffas. Det förvärvade immunförsvaret tar längre tid att mobilisera och kräver att celler "lär sig" vad som är relevant att märka för destruktion och inte. Antikroppar (immunglobuliner) produceras av B celler (vilka ingår i det förvärvade immunförsvaret) men fungerar som markörer för det medfödda immunförsvaret genom att mediera fagocytos och komplement deponering. IgG återfinns i blodplasma och på ytan av B cellerna. Det medfödda och förvärvade immunförsvaret överlappar varandra delvis och sammantaget är immunförsvarets uppgift att fagocytera, markera, och/eller destruera oönskade mikrober. Till de fagocyterande cellerna i kroppen hör bl a neutrofiler och makrofager.

Proteiner

Proteiner bestämmer många av cellers och bakteriers egenskaper. Proteiner har en mängd olika funktioner bl a utgör de cellers byggstenar, medierar transport av t.ex. näringsämnen och gifter, och fungerar som kommunikationsmedel i kontakter med andra celler. Alla proteiner består av aminosyror och har en sk N-terminal del och en C-terminal del. Hos bakterier är den N-terminala delen av ytbundna proteiner orienterad längst bort från bakterieytan. Korta proteiner kallas peptider.

Grupp G streptokocker

Streptokocker är ett samlingsnamn för en grupp bakterier där de enskilda bakterierna är runda och växer i kedjor. Streptokocker indelas i grupper med avseende på förekomst av specifika sockermolekyler på deras yta. De kan även indelas med avseende på om de kan upplösa röda blodkroppar, i en process som kallas hemolys. Den här avhandlingen handlar om grupp G streptokockerna (GGS). Klassificeringen av GGS har varit otydlig och just förekomsten av kolhydraten G som finns på dessa streptokocker återfinns egentligen hos fyra olika species (*Streptococcus dysgalactiae equisimilis*, *S. equisimilis zooepidemicus*, *S. canis* och *S. anginosus*). GGS kan orsaka sjukdomar som halsfluss och svinkoppor men även mer allvarliga tillstånd som blodförgiftning och vävnadsdöd. GGS skiljer sig från de mer välstuderade grupp A streptokockerna (GAS; *S. pyogenes*) på punkter som att GGS anses mer sällan förekommande, mindre virulenta och i sin tolerans för antibiotikan bacitracin. Annars orsakar de en likartad sjukdomsbild med rodnad, svullnad, värmekänsla och smärta vilket kännetecknar inflammation.

M proteinet och protein G

Den typiska virulensfaktorn hos GAS och GGS, är M proteinet. Ett otal studier pekar på dess nödvändighet för bakteriens överlevnad i mänskligt blod. Genom att binda fibrinogen (ett blodburet protein inblandat i bl a blodlevring) rapporteras M proteinet

kunna hämma bl a deponering av komplementfaktorer på bakteriens yta. Komplementfaktorer fungerar som markeringar för blodbanans främsta fagocyter, neutrofilerna. M proteinet medierar också vidhäftning till mänskliga celler och kan aggregera M uttryckande bakterier, vilket kan göra dem mer svårelimnerade. Protein G är ett annat ytprotein som återfinns hos GGS. Det binder effektordelen (Fc) av immunoglobulin G med bred djur- och subklass specificitet, med hög affinitet (se figur 1). Proteiner som sekvens och funktion liknar protein G finns hos andra bakterier. Denna avhandling beskriver ett M protein hos GGS och hur det interagerar med det mänskliga immunförsvaret, främst det medfödda.

Delarbeten I-IV

I **delarbete I** undersöktes om protein G kunde mediera GGS överlevnad i humant helblod. Jag fann att så ej var fallet utan ett GGS M protein kallat FOG var ansvarigt (se figur 3). Jag klonade och framställde olika delar av FOG som lösliga proteiner och fann att bindningen till fibrinogen återfinns i den N-terminala delen. Jag visar att genom att tillsätta lösligt FOG till helblod orsakas aggrering av neutrofilerna. Vid samtidig tillsats av FOG och den FOG-negativa stammen G148, aggregerade neutrofilerna och blev oförmögna att fagocytera G148 vilken utan tillsats av FOG avdödas. Genom att tillsätta de olika klonade delarna av FOG, kunde jag visa att för full överlevnad krävs hela FOG molekylen, så som den uppvisas på bakterieytan. Tillsats av den N-terminala delen (vilken binder fibrinogen) medierade inte överlevnad trots att denna egenskap anses viktig för överlevnad.

Eftersom ytliga infektioner ofta leder till mer allvarliga tillstånd var vi intresserade av att undersöka förekomsten och ev. identitet av en interaktionspartner i mänsklig hud. I **delarbete II** visar vi att FOG binder till kollagen I, ett rikligt förekommande protein i utrymmet mellan kroppens celler, den så kallade extracellulära matrisen. FOG binder till två distinkta regioner i kollagen I, via sin N-terminala del. Vi kunde blockera inbindningen till humana hudsnitt genom lösligt kollagen I och lösligt FOG, men inte lösligt protein G, vilket indikerar att interaktionen sker just mellan FOG och kollagen I. Vi har med framgång visat att FOG fungerar som adhesin till hud från möss och människa, samt att stammar som uttrycker FOG har en fördel i vidhäftning till human hud.

Både protein FOG och protein G kan binda Fc-delen av IgG vid kontakt med blod plasma. Därför finns två olika "populationer" av IgG bundet till bakteriens yta. Vi visar i **delarbete III** att när protein G bundit IgG är bindningsytan för C1q, initiator av komplementsystemets klassiska väg, inte tillgänglig för C1q. Däremot är IgG bundet via FOG, inte blockerat för C1q-interaktion. Vi undersökte relevansen för de två olika IgG populationerna och fann att IgG som bundit via protein FOG kan orsaka oxidative burst (produktion av fria syre radikaler, vilka anses vara direkt eller indirekt skadliga för mikrober) hos neutrofiler medan IgG som bundit via protein G inte längre var kapabelt att orsaka detta respons. Vidare har vi lokaliserat IgGs bindningsyta i FOG. FOG binder till ett brett spektrum av IgG från djur och av olika subklasser av humant IgG, dock inte IgG3.

I **delarbete IV**, undersöker vi om FOG kan klyvas från bakteriens yta av neutrofil elastas och hur frisatt FOG påverkar monocyter. Vi visar att liksom M1, ett M protein från GAS, binder FOG till monocyter och medierar ett uttryck av IL-8 och MIG. Dessa proteiner har kapacitet att locka neutrofiler och T lymfocyter till infektionsplatsen. MIG har antibakteriell effekt och både GAS och GGS avdödas effektivt i saltfri miljö. Däremot i saltkoncentrationer som motsvarar svett och blodplasma men inte saliv, var GGS mer motståndskraftiga mot MIGs effekt än GAS. Även i frånvaro av MIG var GGS mer toleranta mot nämnda saltkoncentrationer och vi tolkar detta fynd som indikation på en passiv virulens hos GGS vilket skiljer dem från GAS.

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