Title
The genetic basis of Streptolysin S (Beta-hemolysin) production in Group A Streptococcus and its role as virulence factor in invasive infection

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The Genetic Basis of Streptolysin S (Beta-Hemolysin) Production in Group A Streptococcus and its Role as Virulence Factor in Invasive Infection

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Pathology

by

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2006
The dissertation of Vivekanand Datta is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2006
DEDICATION

I dedicate my dissertation to my dad Dr. K.C. Datta and my mother Mrs. Mina Datta who have been my constant encouragement throughout my life.
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<tr>
<td>HIF-1$\alpha$</td>
<td>Hypoxia Inducing Factor-1 alpha</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthetase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
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<td>$Lactococcus lactis$</td>
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<tr>
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<td>Luria Brutani</td>
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<tr>
<td>mRNA</td>
<td>messenger ribosomal nucleic acid</td>
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<td>Necrotizing faciitis</td>
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<tr>
<td>ORF</td>
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<td>TNF$\alpha$</td>
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Abstract Presentation

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Manuscript


ABSTRACT OF THE DISSERTATION

The Genetic Basis of Streptolysin S (β-Hemolysin) Production in Group A Streptococcus and its Role as Virulence Factor in Invasive Infection

by

Vivekanand Datta

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2006

Professor Victor Nizet, Chair

Group A Streptococcus (GAS) is an important human pathogen producing a wide array of infections, from self-limited pharyngitis or impetigo to severe, life-threatening invasive conditions such as necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS). Colonies of GAS grown on blood agar media generate a distinctive zone of surrounding clearing known as β-hemolysis. The GAS β-hemolytic phenotype is produced by the action of the potent cytolytic toxin, streptolysin S (SLS). In our earlier work, the genetic locus for SLS production was mapped to a series of nine GAS chromosomal genes (sagA–sagI) resembling the biosynthetic operon for a bacteriocin with sagA encoding the predicted toxin prepropeptide. Targeted plasmid integration defined the functional boundaries of the sag locus, and heterologous expression in Lactococcus lactis defined these nine GAS genes as sufficient for the production of active toxin.
The present study employs precise, in-frame allelic replacement mutagenesis and single gene complementation to demonstrate the individual requirement of \( \text{sagA} \), \( \text{sagB} \), \( \text{sagC} \), \( \text{sagD} \), \( \text{sagE} \), \( \text{sagF} \) and \( \text{sagG} \) for SLS biosynthesis, and to further prove that \( \text{sagE} \) serves an immunity function. Site-directed mutagenesis of specific amino acids in SagA supports the designation of SLS as a bacteriocin-like toxin. Elimination of \( \text{sagA} \) did not produce alterations in the expression of GAS M protein, hyaluronic acid capsule or cysteine protease. Open reading frames bearing homology to the GAS \( \text{sag} \) operon were found to be responsible for production of SLS-like \( \beta \)-hemolysins in isolates of groups C and G streptococci and \textit{Streptococcus porcinus} associated with invasive human infections. Aspects of the bacterial-host interaction in the pathogenesis of GAS infection were next examined using a wild-type invasive serotype M1 GAS isolate and its isogenic \( \Delta \text{sagA} \) mutant. Neutrophil killing functions under the transcriptional control of hypoxia-induced factor-1\( \alpha \) were demonstrated to be an essential component of host defense against GAS infection. SLS was found to contribute to necrotic tissue damage and facilitate systemic infection. Companion tissue culture studies suggest the virulence role of SLS may reflect a combination of cytolytic, antiphagocytic and proinflammatory properties. Inhibition of SLS activity may have potential as an adjunctive therapy in serious GAS infection.
INTRODUCTION

A. **Group A Streptococcus (GAS)**

   In 1874, the Viennese surgeon Theodore Billroth first discovered chain-forming bacteria in the infected wound of a patient and called these microbes “streptococci”, after their beaded appearance (Greek: “strepto” = twisted, “kokkos” = berry). Later the German physician Anton Rosenbach completed the scientific name *Streptococcus pyogenes* in recognition of the characteristic production of pus at the sight of infection. *S. pyogenes* is exclusively a human pathogen seen to form long or short chains when isolated in clinical material. In the microbiology laboratory, it is easily recognizable by its β-hemolytic phenotype (see below) when grown on solid media containing sheep blood cells. In the 1930’s, Rebecca Lancefield developed a serotyping system for classifying the β-hemolytic streptococci based on the group-specific antigen composition of cell wall carbohydrates. *S. pyogenes* was classified as “group A Streptococcus” (GAS), and is the only species represented in this group.

   GAS colonizes up to one third of healthy individuals without producing clinical symptoms. However, GAS is also implicated as the causative agent of a large number of suppurative and non-suppurative infectious diseases. GAS is the most common cause of bacterial pharyngitis, commonly designated as “strep throat”, and is also associated with a variety of skin infections including impetigo and cellulitis. Local extension of pharyngeal infection can result in paratonsillar or retropharyngeal abscesses or cervical lymphadenitis, while deeper spread of skin involvement may
produce the syndrome of erysipelas. The pathogen is also among the common causes of human lymphangitis, sinusitis, pneumonia and musculoskeletal infections.

In addition to its spectrum of acute infections, GAS has also been established as the trigger of a number of immune-mediated post-infectious sequelae, such as acute glomerulonephritis, acute rheumatic fever and reactive arthritis (Bronze and Dale, 1996; Gibofsky and Zabriskie, 1995; Gibofsky et al., 1998). Acute glomerulonephritis is a classic soluble complex-mediated injury to the kidneys characterized by low complement levels, but the particular streptococcal antigen(s) responsible for triggering this pathology have not been clearly defined (Nordstrand et al., 1998). Acute rheumatic fever following streptococcal infection, as diagnosed by the modified Jones criteria (Saxena, 2000), may involve heart, joint, brain or skin tissues. Repeat episodes can lead to severe heart valve damage referred to as rheumatic heart disease (WHO, 2004).

B. Resurgence of invasive GAS infections

Over the last two decades, a substantial increase in reports of severe, invasive forms of GAS infection has been widely appreciated in several regions of the world. In Canada, prospective population-based studies set the annual prevalence of invasive GAS disease at 1-5 cases per 100,000 individuals (Davies et al., 1996). In certain geographic areas, an incidence as high as 26 cases per 100,000 people has been described (Zurawski et al., 1998). In a large published series, based on active population-based laboratory surveillance between 1995 and 1999, O'Brien and colleagues estimated that 9,600-9,700 cases of invasive GAS disease occur in the United States each year, resulting in 1,100-1,300 deaths (O'Brien et al., 2002).
Prominent among the invasive disease syndromes were deep-seated soft tissue infections such as necrotizing fasciitis (NF) and myositis, frequently accompanied by development of streptococcal toxic shock syndrome (STSS) (Davies et al., 1996; Stevens et al., 1989; Stevens, 1992, 2000).

Although invasive GAS infection may strike even previously healthy children and young adults, elderly patients and those with underlying medical conditions are at greater risk, especially for the development of NF (Davies et al., 1996). Predisposing medical conditions include extremes of age (very young or elderly), HIV infection, cancer and diabetes mellitus. In pediatric populations, a strong correlation between the exanthem of primary varicella (chickenpox) and development of invasive GAS infection has been noted (Brogan et al., 1995; Laupland et al., 2000; Mills et al., 1996). The overall mortality of patients with invasive GAS infection is approximately 15%, but this mortality rate nearly doubles in patients greater than 65 years of age. It has further been reported that 1 in 5 cases is may be acquired in the hospital or nursing home settings.

Although recent studies have provided evidence that host immunogenetic factors play an important role in determining the outcome of invasive GAS infections (Kotb et al., 2002), it is unlikely that changes in host factors alone could account for this marked change in epidemiology. The rapid evolution of bacteria suggests a more likely scenario in which certain GAS strains have acquired a repertoire of virulence factor(s) that can, in the susceptible host, trigger severe and potentially fatal diseases. The complexity of GAS strain populations (> 130 serotypes based on the hypervariable
surface bound M protein), the organism’s production of a multitude of extracellular toxins, and the well documented interspecies transmittance of virulence genes via bacteriophages raise the possibility that a major virulence alteration in GAS could underly the increase in invasive infection (Stevens, 1999). Alternatively, decreasing herd immunity against GAS with the advent of improved detection and antibiotic treatment could be another contributing factor to the change in invasive disease epidemiology (Stevens, 1992).

Serological classification based on surface M proteins (and to a lesser extent T antigens) has been employed to study the epidemiology of invasive GAS infection (Efstratiou, 2000). In several studies, strains belonging to serotype M1 have been identified as the single leading cause of severe infections (Chatellier et al., 2000; Cockerill et al., 1998), followed by a limited group of other “invasive serotypes”. In the large Canadian series, Davies reported M1 to represent 24% of total cases, followed by M12 (7.4%), M4 (6.5%), M28 (6.2%) and M3 (5.8%). In published U.S. epidemiologic studies, serotypes M1, M28, M12, M3 and M11 together comprised 49.2% of invasive isolates (O’Brien et al., 2002). One particular clone of serotype MIT1 GAS with increased virulence potential has disseminated globally and persisted for more than 20 years (Aziz et al., 2004).

Epidemiological surveys have linked particular GAS M serotypes to differences in pathogenic potential based on tissue site. For example, an organism producing a superficial skin infection may on occasion give rise to a secondary respiratory infection. In contrast, pharyngeal isolates are rarely found to cause superficial skin infection
(Bessen et al., 1996). Serotypes M1, M12, M28 and M6 have been found to be the most commonly found GAS strains in cases of pediatric pharyngitis (Shulman et al., 2004). Because invasive pediatric GAS strains tend to be identical to acute pharyngitis strains prevalent in the same community settings; the pharynx is felt to represent the major reservoir for invasive GAS disease (Haukness et al., 2002).

C. Necrotizing fasciitis (NF) produced by GAS

NF is the most common and best recognized of invasive GAS disease syndromes, having gained wide attention in the lay press as the “flesh-eating bacterium”. GAS NF is a rapidly progressive infection where extensive tissue involvement develops over 24-96 hours in association with severe toxicity, multiorgan failure and high associated morbidity and mortality (Efstratiou, 2000). While fulminant forms of NF are generally recognized as acute GAS infections, additional bacterial species can produce NF in isolated or mixed infection, sometimes with a more subacute presentation (Ballon-Landa et al., 2001; Bisno and Stevens, 1996; Brogan and Nizet, 1997; Davies et al., 1996; Owens et al., 1978). NF affects all age groups, but it is much common in people with diabetes, minor or major trauma, or some degree of immunocompromise (Seal, 2001).

The clinical presentation of GAS NF is manifest by the organism spreading through the tissues above the deep fascia resulting in gangrene of subcutaneous fat and dermis. Clinical signs are distinct from other soft tissue diseases like erysipelas and cellulitis that usually present a defined border (Brogan and Nizet, 1997). Initially, NF
presents with an area of localized pain, swelling and erythema (Brogan et al., 1995), which is typically followed by patchy discoloration of the skin with pain and swelling but without a defined margins and lymphangitis (Schwartz et al., 1995). In a short period of time, the overlying skin may develop black patches and bullae. The rapidly progressing bacterial infection in the fascial planes is nearly always associated with streptococcal bacteremia (Bouvet, 2001). Soon the patient may rapidly develop multiorgan failure, acute respiratory distress syndrome, compromised renal function, coagulopathy, liver abnormalities and generalized edema characteristic of STSS (Brogan and Nizet, 1997; Stevens, 2000).

Radiographic evaluation of suspected GAS NF may utilize CT scan or MRI to delineate the extent of the tissue injury (Brogan et al., 1995), while clinical laboratory studies may reveal a leukocytosis with a high percentage of immature forms (median 50% band neutrophils), azotemia, and elevated creatinine kinase corresponding to release from damaged tissues. Prompt surgical intervention is essential to debride necrotic tissues and maximize the chances for survival and functional recovery. Intensive supportive care and intravenous antibiotics are the other cornerstones of therapy. **Fig. 1** includes images of patient with severe GAS NF and TSS (a) immediately after surgical debridement of the affected extremity, and (b) at a later stage of clinical recovery following skin grafting.

Tissue samples collected during surgery are typically sent for laboratory testing including histologic examination under light microscopy to define the extent of necrosis and identify the margin of viable tissue, as well as for Gram stain and culture isolation.
of the causative infectious agents. Microscopic studies of GAS NF reveal severe necrosis involving the subcutis, often with extension to the dermis and epidermis (ulceration) or into the adjacent muscle tissues. Inflammatory cell recruitment may be delayed at the early stages of infection, but ultimately a mixed inflammatory infiltrate with abundant neutrophils is found interspersed among the sheets of bacteria. Vasculitis of the vessels bridging the fascial planes with thrombotic occlusion of the lumen is another characteristic manifestation. Representative histological studies from a case of GAS NF are presented in Fig. 2.

D. Virulence mechanisms of GAS

GAS possesses a wide array of proven and suspected virulence factors that can contribute to human transmission and colonization, as well as each of the stages in the development of invasive disease (Cunningham, 2000). Some of the best-studied GAS virulence factors such as M protein, cysteine protease and hyaluronic acid capsule contribute to disease pathogenesis through multiple mechanisms. An overview of GAS virulence phenotypes is facilitated by classification into the following broad themes: (1) adherence to and invasion of host epithelium, (2) avoidance of immunological clearance, and (3) direct or indirect injury to host cells and tissues.

D1. Adherence and invasion of host epithelium

The earliest step in GAS pathogenesis involves successful colonization of host cells on the pharyngeal or skin epithelium. Here interaction of the bacterial surface
molecules with components of the mammalian cell surface leads to attachment of the bacteria to the host cell. Early studies demonstrated that the surface-anchored M protein promoted GAS epithelial adherence (Ellen and Gibbons, 1972). Isogenic GAS mutants lacking M protein do not adhere to human pharyngeal epithelial cells as efficiently as the wild-type parental strain (Courtney et al., 1994). M-protein also facilitates attachment to keratinocytes during the course of GAS skin infection (Okada, 1994). The human membrane cofactor protein CD46, has been identified as a cellular receptor for M-protein (Okada, 1995), interacting with conserved domains in the C repeat section of the GAS molecule. It is also been reported that the M-protein is essential for the persistence of GAS infection in a rat model (Hollingshead et al., 1993b). Adherence of bacteria via M-protein on to Hep2 cells induces IL-6 expression and stimulates the local inflammatory response (Cunningham, 2000).

In contrast, there are certain cell types in which M-protein does not seem to represent the major surface adhesin. For example, elimination of M-protein did not alter the adhesion of GAS to buccal epithelial cells (Caparon et al., 1991). Here GAS binding appeared to be mediated via a completely different surface anchored molecule: lipoteichoic acid (LTA) (Beachey and Ofek, 1976). Fibronectin in the extracellular matrix was found to be the host factor to which the LTA was binding (Simpson, 1983). In these studies, antibody against LTA blocked the adhesion of GAS the epithelial cells, whereas pretreatment of the target cells with LTA had the effect of saturating receptors and reducing bacterial adhesion. LTA itself interacts with other GAS cell surface molecules including the M protein, as the anionic backbone composed of polyglycerol
phosphate is attracted to positively charged residues on these proteins (Ofek et al., 1982).

Other GAS surface proteins including protein F (aka Sfb) (Hanski et al., 1992), the serum opacity factor (Kreikemeyer et al., 1995), and SfbX (Jeng et al., 2003) and collagen-binding proteins (Kostrzynska et al., 1989; Visai et al., 1995) contribute to a large arsenal of extracellular matrix binding factors for the pathogen (Courtney, 1999). The GAS surface hyaluronic acid capsule can also facilitate bacterial adherence via interactions with collagen (Dinkla et al., 2003) or the hyaluronate receptor CD44 in epithelial cell membranes (Schrager et al., 1998). Attachment to the eukaryotic cell is establishes colonization of the host and is a prerequisite for the initiation of tissue penetration and the initiation of invasive disease.

In the pathogenesis of invasive infection, GAS intracellular invasion of human epithelial cells may play a pivotal role. Two theories have been put forth for the role that GAS access into the intracellular compartment plays in the pathogenesis of invasive infection. First, an equilibrium in which some GAS exists intracellularly may provide a haven from certain components of immune clearance leading to persistent infection. Among the 30% of the population that is subject to asymptomatic throat carriage of GAS, and it has been reported that those strains isolated from chronic carriers invade pharyngeal epithelial cells with greater efficiency (Molinari and Chhatwal, 1998). The second theory advocates that internalization of GAS is the essential step towards deeper tissue invasion by the bacteria (LaPenta et al., 1994). The rate of internalization of GAS by human respiratory epithelial cells is similar to the
invasion frequency of well-established intracellular bacterial species such as *Listeria* and *Salmonella* (LaPenta *et al.*, 1994). The globally disseminated clone of M1T1 serotype associated with invasive infections is particular adept at high frequency epithelial cell invasion (Cleary *et al.*, 1998). The uptake of M1 GAS reflects host cell cytoskeletal rearrangements induced during the invasion process (Dombek *et al.*, 1999).

Studies with isogenic GAS mutants indicate that the process of intracellular invasion is aided by the M-protein itself (Cue *et al.*, 1998), serum opacity factor (Timmer *et al.*, 2005), and extracellular matrix binding proteins such as SfbI (Molinari *et al.*, 2001). Binding to fibronectin appears to trigger invasion through the α5β1 integrin receptor on the epithelial cell surface (Kreikemeyer *et al.*, 1995). Finally, studies of bacterial translocation in two human skin models indicate that Rae1-dependent cell-signalling triggered by interaction of the GAS hyaluronic acid capsule with CD44 popened intercellular junctions and promoted tissue penetration by the bacterium through a paracellular route (Cywes and Wessels, 2001).

**D2. Avoidance of immunological clearance**

A key component of human host defense against GAS is phagoecytosis clearance mediated by host neutrophils and macrophages. GAS in turn expresses a number of phenotypic traits to mitigate phagoecytosis clearance, chief among which is the surface bound M protein (Fischetti, 1991). Several distinct mechanisms have been proposed to explain the antiphagoecytic behavior of M-protein. The most simplistic mechanism involves the simultaneous binding of serum factor H to the M protein conserved C repeats and the factor H-like protein (HPL) to the M protein hypervariable region
(Bisno et al., 2003; Fischetti, 1991; Johnsson et al., 1998). The nonopsonic binding of factor H and HPL inhibits the activation of complement pathway by interfering with the deposition and activation of C3b. Human C4b-binding protein, another component of the complement control system, has also been shown to bind the hypervariable region of M-protein and hamper phagocytic clearance (Berggard et al., 2001). Finally, binding of fibrinogen to the hypervariable domain also appears to block complement activation and decreases neutrophil phagocytosis (Horstmann et al., 1992).

Antibodies against the serotype specific M-protein domains are sufficient to overcome the protective effect of M-protein and facilitate successful opsonophagocytosis (Bisno et al., 2003). While the prevailing hypothesis held that M1 is antiphagocytic, the actual internalization of GAS into host immune cells was not well understood. In a recent study that provided new insight, investigators studied phagocytosis by human neutrophils of wild-type GAS strains and isogenic mutants deficient in expression of M protein and/or the M-like protein H (Staali et al., 2003). Their findings demonstrated that both WT and M protein-deficient GAS were both easily phagocytosed after activating actin polymerization in the host PMNs (Staali et al., 2003). However, the presence of M-protein or M-related protein on the GAS proved to be important for their intracellular survival after phagocytic uptake and consequently their capability to multiply in whole blood (Staali et al., 2003). Although recruited neutrophils are a major component of innate immunity against any invading pathogen, resident tissue macrophages represent a primary line of host phagocyte defense. The protective function of resident macrophages against GAS also appears to
depend on their ability to efficiently take up and kill the bacterium (Goldmann et al., 2004).

Additional GAS surface proteins related to M-protein have been identified encoded by the *emm* or *emm*-related genes. These proteins are grouped under the umbrella of “M-like proteins”, and include immunoglobin (Ig)-binding proteins that have a synergistic role along with M-protein to evade phagocytosis (Ji et al., 1998; Podbielski et al., 1996). More than twenty genes have been identified in the M-like superfamily, sharing almost 70% sequence homology at the 5’ end (Hollingshead et al., 1993a). All members of the M-protein superfamily has three distinctly conserved domains, including include the protein anchoring H-domain, the peptidoglycan-associated domain and cell-wall binding domain (Hollingshead et al., 1993a). Immunoglobulin-binding proteins that are encoded by a *emm* related genes, interact with Igs outside their antigen-combining site, within the Fe region (Boyle, 1998; Pack and Boyle, 1995a). GAS have been shown to express type II Fe-binding receptors on their cell surface, which bind to different class of IgG with varying affinity (Pack and Boyle, 1995b). The Ig-binding proteins have been shown to function as virulence factors in murine air-sac model and skin infection models (Boyle et al., 1998; Schmidt et al., 1997). Expression of IgG binding proteins are also associated with resistance to phagocytosis, enhanced bloodstream survival, greater degrees of invasive tissue infection (Podbielski et al., 1996; Raeder and Boyle, 1993a, b). An association between differences in IgG binding protein and severity of invasive disease produced by M1 strains has been established (Raeder and Boyle, 1995, 1996), and it is further
known that bacterial passage *in vivo* may provoke the change in the IgG-binding protein type (Raeder and Boyle, 1996). This conversion of the IgG binding protein type was attributed to the post-translational modification by the GAS cysteine protease SpeB (Raeder *et al.*, 1998). Apart from the IgG binding proteins, GAS also express a protein with high affinity for human IgA (Lindahl and Akerstrom, 1989; Stenberg *et al.*, 1992). The IgA binding protein serves an antiphagocytic function (Husmann *et al.*, 1995), and high IgA-binding protein activity levels have been observed in GAS isolated from wounds and deep tissues (Bessen and Fischetti, 1990).

Strains of GAS rich in surface expression of hyaluronic acid capsule have also been recognized to be more resistant to phagocytosis (Fischetti, 1991; Foley and Wood, 1959; Moses *et al.*, 1997). The GAS capsule is invariably composed of a polymer of hyaluronic acid containing repeating units of glucuronic acid and N-acetylglucosamine (Stoolmiller and Dorfman, 1969). Polymeric capsule synthesis involves a three-gene operon: *hasA*, *hasB* and *hasC*, which is under the control of a negative regulatory system CsrR, influencing capsule formation (Dougherty and van de Rijn, 1992). GAS mutants lacking their capsules are highly susceptible to phagocytic clearance, and compared to their wild-type counterparts, are decreased in their colonization capacity of in an animal model (Husmann *et al.*, 1997; Schmidt *et al.*, 1996; Wessels and Bronze, 1994). While the antiphagocytic properties of the hyaluronic acid capsule and their role in promoting invasive GAS disease in human are evident (Johnson *et al.*, 1992), the antibodies raised against the capsule do not increase opsonic clearance of the bacteria (Dale *et al.*, 1996). The mechanism of resistance to phagocytosis conferred by the
hyaluronic acid capsule appears to be a physical barrier function preventing access of phagocytes to opsonic complement proteins bound to the bacterial surface (Cunningham, 2000). Hyaluronic acid capsule (along with M-protein) where shown to be important for the development of GAS necrotizing fasciitis in the mouse model (Ashbaugh et al., 1998).

Host activation of C5a is sufficient to produce a pro-inflammatory reaction and stimulate chemotaxis of neutrophils to the site of infection, facilitating GAS elimination. The GAS produce a surface C5a peptidase that proteolytically inactivates human C5a, thereby blocking the chemotactic movement of neutrophils (Ji et al., 1996; Wexler et al., 1985). In mammals, endogenous expression of cationic antimicrobial peptides provides an additional innate barrier of defense against invasive GAS infection (Nizet et al., 2001). These peptides are expressed by epithelial cells including skin keratinocytes, as well as phagocytic cell types such as neutrophils and macrophages. Mice deficient in the cathelicidin class of antimicrobial peptides show markedly enhanced susceptibility to invasive GAS skin infection and a neutrophil defect in GAS killing (Nizet et al., 2001). The human homologue of the murine cathelicidin is called LL-37, and invasive M1 strains of GAS appear to have evolved a number of mechanisms to avoid LL-37 killing. The streptococcal inhibitor of complement-mediated lysis (SIC) was is expressed by many invasive M1 strains of GAS, and is a component of the mga regulon expressed maximally in logarithmic growth phase and can inhibit the complement membrane attack complex (Akesson et al., 1996; Hoe et al., 1999; Mejia et al., 1997; Stockbauer et al., 1998). SIC interacts with and inhibits the
antimicrobial peptides LL-37 and α-defensin, potentially promoting the growth of GAS in the host (Frick et al., 2003). Alteration of GAS cell surface to incorporate positively charged modification may be one other mechanism of GAS avoidance of antimicrobial peptide clearance. In this respect, we have shown that teichoic acid D-alanylation increases GAS resistance to host AMPs (Kristian et al., 2005).

D3. GAS-mediated injury to host cells and tissues

Several GAS extracellular products may play a significant role in disease pathogenesis. GAS produce a hyaluronidase that may degrade hyaluronic acid of the connective tissues, promote liquefaction of pus, and allow the spread of the pathogen through tissues planes (Baker et al., 2002). Streptokinase, a secreted plasminogen-binding protein, is a known to dissolve fibrin clots, converting plasminogen to plasmin on the bacterial cell surface (Ashbaugh et al., 1998). Although the role of streptokinase is better studied in models of post-streptococcal glomerulonephritis (Nordstrand et al., 1998), a recent study suggests it may play a key role in invasive GAS disease (Rezcallah et al., 2004). M1 GAS isolated from the spleens of a lethally-infected mice demonstrated a high level of streptokinase compared to the unpassaged strain (Rezcallah et al., 2004). This effect was due to a combination of increased expression of the streptokinase gene (skh) gene and decreased expression of streptococcal cysteine proteinase, which efficiently degrade streptokinase (Rezcallah et al., 2004).

The role of the cysteine protease SpeB in GAS disease is complex and multifactorial. Early studies showed decreased mortality in mice challenged with
SpeB-negative mutant strains, and that these strains were also more susceptible to phagocytic clearance (Lukomski et al., 1998; Lukomski, 1997). Examining the pathology of the affected soft tissues, necrosis of feeding blood vessels and surrounding tissue appeared to be associated with the dissemination of the GAS from the initial site of infection (Lukomski, 1999). Neutralizing antibodies against SpeB may protect against severe GAS disease, as evident in studies in which mice were immunized with recombinant SpeB and showed prolonged the survival upon subsequent challenge (Kapur et al., 1994; Kuo et al., 1998). Other investigators, however, have failed to confirm a critical role of cysteine protease in the development of tissue necrosis and dissemination of GAS in a mouse necrotizing fasciitis model (Ashbaugh et al., 1998). GAS cysteine protease cleaves host targets such as the extracellular matrix proteins fibronectin and vitronectin (Kapur et al., 1993b), as well as processing human IL-1β into its active form (Kapur et al., 1993a). However, SpeB has also been implicated in cleaving the N-terminal end of the M-protein from the GAS cell surface (Kansal et al., 2000) and degrading a variety of its other secreted proteins (Aziz et al., 2004), potentially neutralizing some of the virulence potential of the pathogen. Indeed, epidemiologic studies have found that 41% of M1 GAS isolates from GAS of streptococcal toxic shock syndrome (STSS) produced little or no SpeB, compared to only 14% of isolates recovered in GAS bacteremia without STSS (Kansal et al., 2000).

STSS characterized by hypotension and leading to multiorgan failure was first described in detail by Stevens (Stevens et al., 1989). The pathogenesis of STSS has at its root the activities of several GAS extracellular pyogenic exotoxins (SpeA, SpeC,
SpeF, SpeG, etc.) and streptococcal superantigens known for their pyrogenicity and superantigenic effects on immune cells (Mollick et al., 1993; Norrby-Teglund et al., 1994a; Norrby-Teglund et al., 1994b). Superantigens are molecules that are capable of binding to the VB chain of the T-cell receptor (TCR) and simultaneously to the MHC class II molecule expressed on the antigen-presenting cell (e.g., monocytes or dendritic cells). This enhanced cellular interaction leads to excessive immunostimulatory cell proliferation and a cascade of unregulated inflammatory cytokine production (Kotb, 1995). STSS may result from a massive induction of cytokines IL-1, IL-6, TNFβ, and INFγ (Kotb, 1992; Schlievert, 1993). M1 is the most common GAS serotype encountered in most cases of STSS, and a particular subclone of M1 GAS has disseminated globally in association with severe infections (Davies et al., 1996).

It has been reported that patients who succumbed to STSS caused by this M1 strain have developed only very low titers of anti-M1 antibodies (Holm et al., 1997). In a recent study M1 protein-fibrinogen complexes were found to activate neutrophils and mediate vascular leakage (Herwald et al., 2004). Increased vascular permeability is a pathophysiological mechanism during shock that leads to hypotension and decreased tissue perfusion. M1 protein-fibrinogen complexes crosslink small β2 integrins to activate neutrophils to release heparin binding protein (Herwald et al., 2004). In fact, subcutaneous injection of GAS in mice leads to the vascular leakage in the lungs as a result of complex deposition, even though the bacteria themselves do not appear into the bloodstream (Herwald et al., 2004).
E. Clinical microbiology of GAS

In patient samples collected from mucosal or skin colonization, inflammatory exudates, deep pus collections, or bloodstream spread, GAS appear as Gram-positive chains under light microscopy. When cultured on blood agar plates, the production of a characteristic zone of complete red blood cell clearance termed β-hemolysis (Fig. 2) is an important clue in differentiating GAS from many other streptococcal species which possess a greenish surrounding zone (α-hemolysis) or fail to produce hemolysis at all. Originally described by Lancefield, the β-hemolytic streptococci can be divided into several groups based on their antigenic differences by group-specific polysaccharides located in the bacterial cell wall (Koneman, 1997). More than 20 serologic groups have been identified and designated by letters: A, B, C, etc. Among all the Lancefield groups, GAS and GBS are the most important human pathogens, but clinically important infections can also be seen with GGS, GCS or GFS. The group A carbohydrate antigen is composed of carbohydrate N-acetyl-β-D-glucosamine linked to rhamnose polymer backbone (Cunningham, 2000). Although serologic grouping by the Lancefield method is the criterion standard for differentiation of pathogenic streptococcal species, GAS can be identified more cost-effectively by any of a number of latex agglutination, co-agglutination, or enzyme immunoassay procedures.

GAS is further differentiated serologically by M-protein, the surface protein that can be extracted from the bacteria after boiling in hydrochloric acid. Classification of clinical isolates for epidemiological studies solely dependent serology of surface M-protein with available polyclonal sera is often faulty and possess technical challenges to
even experienced investigators. Recently, Beall and colleagues at the Centers for Disease control have developed PCR-based emm gene sequencing as a reliable method to distinguish between different M-protein strains (Beall et al., 1996).

Another aid to distinguishing GAS strains from other β-hemolytic streptococci is their intrinsic sensitivity to bacitracin. A disc containing 0.04 U of bacitracin inhibits the growth of more than 95% of GAS, whereas 80-90% of non-group A strains are resistant to this antibiotic. The bacitracin disc test is simple to perform and interpret in an office-based laboratory and is sufficiently accurate for presumptive identification of GAS. Presumptive identification of a strain as GAS can also be made on the basis of production of the enzyme L-pyrolidonyl- β-naphthylamide (PYRase). Among the β-hemolytic streptococci isolated from throat culture, only GAS produce PYRase, which can be identified on the basis of the characteristic color change (red) after inoculation of a disk on an agar plate followed by overnight incubation.

F. Streptolysin S and GAS β-Hemolysis:

GAS produces two hemolysins known to damage erythrocyte membranes, streptolysin S (SLS) and streptolysin O (SLO). The designations reflect the observation that SLS is oxygen-stable while SLO is oxygen-labile. Although the exact biology of neither hemolysin has been thoroughly elucidated, SLO has been the better studied of the two. SLS is a 57 kDa protein whose encoding gene has been cloned and characterized (Kehoe and K.N.Timmis, 1984). SLO is expressed and secreted by almost all GAS clinical isolates and related streptococci in groups C and G (Kehoe and
K.N. Timmis, 1984). Because of its oxygen-sensitivity, SLO does not produce detectable hemolysis on the surface of blood agar plates, but can be appreciated in “stabs” into the agar where oxygen tension is reduced. SLO belongs to the family of thiol-activated bacterial cytotoxins (Alouf, 1980), whose characteristics include inhibition by cholesterol and oligomerization in the erythrocyte membrane to form a pore. SLO is highly immunogenic and an elevated level of serum anti-SLO antibodies is a useful clinical measure for recent GAS infection (Cunningham, 2000). There is recent evidence SLO may play a role in virulence by protecting GAS from phagocytic clearance and in a murine sepsis model (Limbago et al., 2000; Sierig et al., 2003), but an individual virulence role is not apparent in the GAS model of necrotizing skin infection (Fontaine et al., 2003; Limbago et al., 2000).

The hallmark β-hemolytic phenotype of GAS on blood agar (Fig. 3) is the byproduct of the SLS toxin. In contrast to SLO, SLS does not engender antibodies during the course of natural infection. Despite nearly a century of investigations, SLS has never been fully purified and remains one of the great enigmas of GAS biology. By weight, SLS is regarded as one of the most potent cytoxins known (Koyama, 1963), with a cytolytic spectrum that includes not only erythrocytes but other eukaryotic cells such as lymphocytes (Hryniewicz and Tagg, 1977), neutrophils (Ofek et al., 1972), tissue culture cells (Takeo and Takeo, 1966) and subcellular organelles as lysosomes (Bernheimer and Schwartz, 1964) , and mitochondria (Keiser et al., 1964). SLS can exist in intracellular, cell surface-bound and extracellular forms (Ginsburg and Grossowicz, 1958). Attempts at cell-free preparation of SLS require a carrier molecule
such as yeast RNA core or albumin to stabilize the cytolytic activity of SLS within the culture supernatants (Calandra and Cole, 1981). Cholesterol does not inhibit SLS-mediated hemolysis, but rather the toxin’s activities can be blocked by trypan blue and certain phospholipids (Ofek et al., 1970).

Koyama calculated the molar ratio of SLS to oligonucleotide in partially purified SLS-RNA core preparations to be 0.3. Gel filtration analysis of such preparations yielded a M.W. of 12.0 kD for the SLS oligonucleotide complex, suggesting the mature toxin polypeptide moiety could be approximately 0.3/1.3 x 12,000 or 2.8kDa in size (Bernheimer, 1967). Attempts in sequencing the polypeptide moiety were unsuccessful, suggesting that the amino terminus could be blocked or that the peptide moiety was cyclical in nature. The mechanism by which SLS damages intact cells was early on attributed to alterations in the permeability of cell membrane systems (Bangham et al., 1965). Addition of exogenous phospholipids inhibited this effect, so it has been hypothesized that SLS interacts with and modifies cell membrane phospholipids (Ginsburg, 1972. However, SLS does not possess measurable phospholipase activity (Elias, 1966 #643), nor does electron microscopy reveal the type of membrane holes seen with SLO (Kehoe, 1984). Analysis of the hemolytic activity of partially purified SLS extract indicates that the mechanism of hemolysis is similar to that of complement-mediated hemolysis (Carr et al., 2001). SLS in a soluble, functionally active form binds and inserts into the cell membrane forming a transmembrane channel that is both time and temperature sensitive (Carr et al., 2001). The stable transmembrane pore in the lipid bilayer is followed by osmotic disruption,
which appears to be the mechanism underlying the SLS mediated hemolysis (Carr et al., 2001). In these studies, osmotic disruption of erythrocytes could be blocked by addition of an osmotic blocker.

Because of its potent cytolytic activity and apparent non-immunogenicity, there has been extensive speculation over the years that SLS may play an important role in the pathogenesis of direct tissue injury in human disease. Marmorek, on first describing streptococcal \( \beta \)-hemolysis in 1895, suggested a direct relationship between virulence and hemolytic power. In the 1970’s, a genetically undefined SLS-negative chemical mutant generated by treating GAS with nitroguanide was observed to be less virulent compared to its wild type non-treated counterpart (Owens et al., 1978). It was hypothesized that a potent cytotoxin such as SLS could act synergistically with hydrogen peroxide and proteases, such as those released by activated neutrophils, to damage membranes of epithelial and endothelial cells (Ginsburg and Varani, 1993), and collectively contribute to the pathogenesis of GAS necrotizing tissue infections. (Ginsburg et al., 1999).

G. Discovery of the genetic basis for streptolysin S (SLS) production by GAS

A major advance in our understanding of the SLS toxin and its potential genetic basis was first achieved in 1998 by Betschel et. al. who applied random transposon integration to create libraries of GAS mutants and screen for loss of the \( \beta \)-hemolytic phenotype produced by SLS. The transposon insertion site in two separate Tn916 mutants, one each in the M1 and M18 serotype backgrounds, was mapped to a
promoter motif upstream of a unique open reading frame (ORF) of 53-aa that was named *sagA* for “streptolysin-associated gene A” (Betschel et al., 1998). These transposon mutants showed decreased virulence in a mouse model of invasive infection, corroborating a potential role for the toxin in disease pathogenesis. This study further set the groundwork for the discovery and elucidation of a complex genetic operon in the GAS chromosome involved in SLS production – studies in which I played an important role prior to initiation of my dissertation studies. The findings of these studies were published in 2000 in the journal Infection and Immunity and are summarized in the ensuing paragraphs.

Plasmid based Tn917 mutagenesis was used as a complementary technique to identify an SLS-negative mutant in the M49 serotype GAS background (Fig. 4A). The site of Tn917 insertion in the GAS chromosome was excised and cloned for sequence analysis. The identified sequence matched completely with an uncharacterized open reading frame (ORF) within a contig of the then ongoing M1 GAS genome project at the University of Oklahoma. Utilizing PCR-based techniques of chromosomal walking, we found that novel ORF was situated a short distance downstream of the *sagA* ORF, and that ultimately a series of 9 contiguous genes could be identified in this chromosomal locus. Consequently, the like “streptolysin-associated gene” terminology was applied to each putative ORF of the locus *sagA, sagB, sagC*, etc. continuing through to *sagI* (Fig. 4B). A strong consensus promoter was observed upstream of *sagA* (but not elsewhere), as well as the presence of rho-independent terminator motifs between the *sagA* and *sagB* ORFs as well as 50 bp downstream of *sagI* are noted.
Sequence homologies of the sag locus genes indicated that SLS was related to the bacteriocin class of antimicrobial peptides (Fig. 4C). The bacteriocins, including the colicins, microcins, lantibiotics, and nonlantibiotic bacteriocins, classically possess antimicrobial activity against closely-related bacteria but can sometimes exhibit broader hemolytic or cytolytic properties (de Vos et al., 1995; Nes and Tagg, 1996). Examples include nisin of Lactococcus lactis (Gross and Morell, 1971) and the Enterococcus faecalis plasmid-encoded hemolysin-bacteriocin (Gilmore et al., 1994). Bacteriocins are ribosomally synthesized as a prepropeptide, comprised of an N-terminal leader sequence of 23-36 residues in length and a propeptide of 22-60 residues in length. Bacteriocins specific posttranslational modifications like dehydration of target amino acids, particularly serine, threonine, and glycine of their propeptide region (Jung, 1991). Modified amino acids are subject to form thioether bond with adjacent cysteines, which transforms these linear propeptides into specialized cyclical bacteriocin molecules with antimicrobial activity (Sahl and Bierbaum, 1998). The modified prepropeptide are translocated to the cell surface by the aid of ATP-binding cassette transporter complex, in most cases the leader peptide is cleaved soon after its translocation to bacterial cell surface (de Vos et al., 1995).

Sequence homology demonstrated sagA to be the candidate structural gene for a bacteriocin-like prepropeptide. In particular, SagA possesses a Gly-Gly sequence motif known to precede the cleavage site in several bacteriocins (van Belkum et al., 1997) (Fig. 5). Cleavage of SagA prior to G-G cleavage site would release a 23-residue leader sequence leaving a 30-residue propeptide. The predicted molecular weight of
the propeptide = 2.9 kDa. The sequence of the 30-aa SagA propeptide is rich in amino acids cysteine, serine, glycine, and threonine; these residues are sites for posttranslational modification in several bacteriocin like molecules (Nizet et al., 2000). The cyclical thioether bond formation and N-terminal presence of 2-oxobutyryl group as seen in other bacteriocins (Meyer et al., 1994), may be part of the reason that SLS peptide sequenceing failed in the past (Alouf and Loridan, 1988).

The SagA propeptide has homology (37% identity, 57% similarity) to MebA, the structural gene product of microcin B17 (Fig. 5) (Nizet et al., 2000). Posttranslational modification of preMebB17 converts glycine, cysteine, and serine residues present in MebA into four thiazole and four oxazole rings found in mature microcin B17 (Li et al., 1996). The translational product of mcbA, is modified into proMecB17 by the action of a tri-peptide enzyme Microcin B synthetase comprised of McbB, McbC, and McbD found in the microcin B17 genetic locus (Madison et al., 1997) and an ABC transporter (encoded by mcbE and mcbF), this ABC transporter is also provides immunity to the E.coli against its own microcin B 17 (Moreno et al., 2002). Sequence homology of SagB with McbB, suggests SagB may be involved in posttranslational modification of amino acid residues in the SagA propeptide on the way to mature SLS production.

SagC, a 40.3 kDa protein with membrane-spanning segments at residues 215-230 and 272-292 as predicted by TopPred II 1.2 has no GeneBank homologies (Nizet et al., 2000). Similar bioinformatics programs were used to determine that SagG possessed six membrane-spanning regions dispersed across its length, which shared
significant homology to an ABC transporter protein (Yamamoto et al., 1996). Bacteriocin like molecules are exported to the bacterial cell surface by ABC transporters (van Belkum et al., 1997). The modified prepropeptide translocated on to the cell surface is also cleaved by a protease, which in many instances is the part of the ABC transport complex (van Belkum et al., 1997). Sequence homology indicates that sagG to sagI encodes for the ABC transporter complex as the part of SLS producing genetic machinery, with SagG containing the ATP-binding cassette (Nizet et al., 2000). SagE encoding for a 25.4 kD probable membrane protein has weak homology to a putative Lactobacillus plantarum bacteriocin immunity protein PlnP (Diep et al., 1996).

Since virtually all strains of GAS produce SLS, for sagA-I to represent the genetic basis for toxin biosynthesis, the locus should be conserved across GAS serotypes. Southern blot analysis was performed on chromosomal DNA digested with SpeI from strains representing 12 differentemmgenotypes. When probed with a fragment encompassing sagD through sagF, two hybridizing bands of expected size were observed in every case, demonstrating conservation of the locus across a broad range of GAS serotypes (Fig. 6).

In order to define the functional boundaries of the sag locus for SLS production, we cloned intragenic fragments of the individual sag ORFs into the conditional plasmid pVE6007 and transformed the wild-type M49 serotype bacterium to generate single-crossover (Campbell-type) plasmid integrational disruptions of each gene (Fig. 7). We observed that plasmid integrations directed into each of the genes produced a nonhemolytic phenotype, while targeted plasmid integrations upstream of sagA or
downstream of sagI had no effect on the SLS phenotype (Nizet et al., 2000). Although these targeted mutagenesis studies verified the association of the sag locus with SLS production as predicted from the transposon mutagenesis and sequence homologies, they fail to demonstrate the unique requirement of individual sag genes because of potential polar effects on downstream gene transcription produced by the large disrupting plasmid (Nizet et al., 2000).

Having demonstrated that the sag locus as a whole is a required for SLS production, we next asked whether these 9 genes were sufficient for production of the mature toxin. The entire nine-gene sag locus was amplified and cloned into an expression vector and transformed in a nonpathogenic, nonhemolytic Gram-positive cocci Lactococcus lactis (Kuipers et al., 1993), transformants were β-hemolytic (Fig. 8) (Nizet et al., 2000). We conclude with that the sag locus is necessary for SLS activity in GAS and all the 9 genes of the locus is sufficient enough to confer the β-hemolytic phenotype to a heterologous, nonhemolytic bacterial species. Heterologous expression of SLS in L.lactis also indicated that the sag locus consists of structural gene encoding for SLS toxin, the genes for modification of the prepropeptide and transport of the toxin to the cell surface.

In our earlier study we discovered the transcription of sagB to sagI as a polycistronic message along with sagA, (Fig. 9). Polycistronic mRNA is a common feature of bacteriocin producing molecules. A rho-independent terminator sequence follows sagA which would correspond with previously reported 450-bp sagA transcript (Betschel et al., 1998). The abundance of mRNAs of structural gene, in contrast to the
downstream genes by virtue of regulatory control of the rho-independent leaky termination appears to be a standard feature of bacteriocins (Skaugen et al., 1997). A large transcript of the structural gene SagA prepropeptide and limited transcription of downstream genes products is a wise utilization of biochemical resources by the bacteriocin producing bacteria, by generating large quantity of toxin in the prepropeptide form and reusing the gene products to modify, export the mature toxin.

**H. Central hypothesis and objectives of dissertation research**

As reviewed above, β-hemolysis produced by the exotoxin SLS is a hallmark phenotype of the major human pathogen GAS. The potency and broad spectrum cytolytic action of the SLS toxin suggest it could play a role in the pathogenesis of invasive GAS infection, a finding supported by studies using SLS- or transposon mutants (Betschel et al., 1998). Our published Infection and Immunity study (Nizet et al., 2000) described the discovery of a 9-gene GAS operon (sagA-I) that was necessary and sufficient for production of SLS. Homologies of sag gene products suggested SLS was a small bacteriocin-type peptide, encoded in the sagA ORF in precursor form, then subsequently modified and exported to the cell surface by downstream genes in the operon. The discovery of the genetic locus for SLS biosynthesis set the stage for a detailed analysis of the independent contribution of individual genes in the operon to SLS production, and for generation of precise genetic reagents to study the virulence functions of the toxin using tissue culture and small animal model systems. Such molecular genetic and function studies represent form the of my dissertation research.
The central hypotheses of my dissertation can be stated as follows:

(A) I hypothesize that SLS is a small peptide cytotoxin produced by the 9-gene sag locus of GAS and that each and every gene component of this locus is required for the proper biosynthesis of the SLS toxin.

(B) I hypothesize that the SagA gene product is the precursor for the SLS toxin and will possess certain signature amino acids essential for cytolytic activity.

(C) I hypothesize that the SagE gene product is required for GAS self-immunity to the toxic effects of SLS.

(D) I hypothesize that SLS is a critical virulence factor in the pathogenesis of necrotizing fasciitis and systemic infection produced by M1 serotype GAS.

(E) I hypothesize that GAS production of SLS exerts virulence effects through a combination of direct cell injury and proinvasive, antiphagocytic and proinflammatory properties.

(F) I hypothesize that normal host phagocytic functions is critical to control of GAS NF produced by SLS-expressing GAS strains.

(G) I hypothesize that other β-hemolytic streptococci associated with invasive human infections may possess SLS-toxins encoded by sag operon homologues.
Finally, I hypothesize that inhibition of SLS activity may decrease GAS virulence potential and represent a novel form of therapy against invasive infections such as NF.

My hypotheses will be tested using a combination of molecular bacterial genetics and testing using \textit{in vitro} and \textit{in vivo} model systems of disease pathogenesis. Precise, in-frame allelic replacement of individual \textit{sag} operon ORFs will be coupled with single gene complementation to unambiguously define the requirement of individual genes for SLS production. Site-directed mutagenesis will be used to probe the requirement of individual SagA amino acid residues for toxin activity. An isogenic SLS mutant will be generated in the M1 serotype background to probe its role in the pathogenesis of GAS NF and systemic dissemination. The cytolytic, proinvasive, proinflammatory, and antiphagocytic properties of SLS will be tested using human tissue culture studies with keratinocytes and purified neutrophils. The murine model of NF will be used to determine the role of SLS in GAS pathogenesis, the role of the master phagocyte regulator HIF-1\(\alpha\) in host defense against the pathogen, and the potential of SLS inhibition to mitigate disease progression.
Fig. 1. Necrotizing fasciitis produced by Group A Streptococcus. Patient is a 40 year old previously healthy man who suffered a minor injury to the left elbow one week prior to admission. Patient presented with excruciating pain, high fever, redness and swelling of the arm and had marked hypotension. (A) Immediate post-operative image following fasciotomy and debridement. (B) Wound several weeks after closure and skin grafting. Copyright obtained from Drs. Allon Moses and Emmanuel Hanski, Hebrew University, Jerusalem.
Fig. 2. Necrotizing fasciitis in the leg of a 17 y-o female caused by group A streptococci. (A) Streptococcal cellulitis provokes intractable skin ulceration. Massive necrosis of the skin and subcutaneous tissue is evident. (B) Gangrenous change is evident in the soft tissue obtained by debridment. (C) Peripheral blood after microbial culture in the same case show short chains of Gram-positive cocci are morphologically typical of Streptococcus. Images are from the website department of pathology, Fujita Health University School of Medicine Japan. http://www.yamagiku.co.jp/pathology, copyright obtained.
Fig. 3. β-Hemolytic group A streptococci. (A) Group A streptococcus seen from the top of the lid. (B) Bacterial colony surrounded by a zone of β-hemolysin seen from the bottom of the Petri dish. Images are from the website department of Pathology, Cambridge University, copyright obtained.
Fig. 4. Genetic locus for Streptolysin S production by Group A Streptococcus. Transposon mutagenesis followed by chromosomal walking to isolate the genetic locus for streptolysin S associated genes (sag) in GAS. (A) M1 and M18 Tn916s mutant and NZ131(M49) Tn917 mutant exhibit an SLS-deficient phenotype when screened on SBA. (B) Map of the sag genetic locus required for SLS production by GAS. (C) Predicted translated products of the genes in the GAS sag-locus, describing their predicted PSORT localization and GenBank homologies. Copyright obtained from ASM press.
Fig. 5. Amino acid sequence similarity between SagA and McbA of *E. coli*. The sagA ORF encodes a 53 aa product with aa sequence features suggestive of a bacteriocin precursor. Features include PGG cleavage site, which would yield a 23 aa leader peptide and a 30 aa propeptide. The Fig. shows similarity of N and C-termini of SagA and bacteriocin microcin B17 precursor McbA. Copyright obtained from ASM press.
Fig. 6. Southern blot analysis showing the conservation of the *sag* locus among GAS isolates from variety of *emm* (M protein) genotypes. Chromosomal DNA from NZ131 and 11 other *emm* genotypes were digested with SpeI. When probed with *sagD* to *sagF* PCR amplicon gave two hybridizing bands. Copyright obtained from ASM press
Fig. 7. SLS phenotype following targeted knockouts of sag locus genes. Targeted plasmid integrational mutants created by homologous recombination event, defined the functional boundary of the sag locus, and the individual requirement of the genes in the expression of SLS. Copyright obtained from ASM press.
Fig. 8. Transcriptional analysis reveals the operon structure of GAS sag locus. RT-PCR analysis of total RNA from NZ131. Transcript of predicted size is identified using a sagA and sagB primer set, sagB and sagC primer set and so on. A large quantity of sagA transcript depicted by the strong signal on northern blot and weaker signals of downstream gene transcription may indicate an elegant method of bioconservation of sag locus. Copyright obtained from ASM press.
Fig. 9. Heterologous expression of the SLS phenotype in *L. lactis*. Plasmid pSagLocus confers b-hemolytic phenotype to non-hemolytic, non-pathogenic gram-positive *L. lactis* subspecies cremoris. Copyright obtained from ASM press.
REFERENCES


CHAPTER I

Genetic analysis of the GAS operon encoding SLS and its role in virulence

A. Introduction

As reviewed in the Introduction, β-hemolysis produced by the exotoxin SLS is a hallmark phenotype used to identify GAS in the clinical microbiology laboratory. Classical biochemical techniques and analysis of partially purified SLS preparations were indicative of a small protein with broad spectrum, potent cytolytic activity. Our previous studies identified the 9-gene sag locus of GAS as both necessary and sufficient for GAS SLS production, mapped the functional boundaries of the locus, and demonstrated its operon structure. Homologies and organization features of the GAS sag operon suggest SLS may resemble a bacteriocin-like toxin. Moreover, the discovery of the genetic basis of SLS production provided a powerful new approach to develop reagents to test the virulence properties of the toxin and its contribution to the pathogenesis of invasive GAS infections such as NF.

This chapter describes a series of molecular genetic and functional studies I conducted to advance our knowledge of SLS biology and its role in GAS disease. The experiments included the following:

(a) The use of precise, in-frame allelic exchange mutagenesis combined with single gene complementation to demonstrate the unique requirement of the sagA, sagB, sagC, sagD, sagF, and sagG genes in SLS production.
(b) Genetic experiments that strongly suggest the *sagE* gene serves an immunity function to protect the GAS bacterium from SLS toxicity.

(c) Site-directed mutagenesis studies which are consistent with the assignment of the *SagA* gene product as a bacteriocin-like peptide.

(d) Precise, in-frame allelic replacement of the *sagA* gene in a virulent M1T1 invasive disease isolate to create and SLS derivative for use in virulence testing.

(e) Phenotypic characterization of the M1T1Δ*sagA* mutant to confirm lack of pleiotrophic effects of capsule, *M* protein or cysteine protease production.

(f) *In vivo* challenges in the murine model to prove that SLS contributes to necrotic skin and soft tissue injury as well as systemic spread of GAS from the initial tissue focus.

(g) Neutrophil and whole blood killing assays to demonstrate that SLS plays a role in GAS resistance to phagocytic clearance.

(h) Cytotoxicity assays to prove that SLS plays a role in injury to human keratinocytes, in synergy with virulence factors *M* protein and SLO.
Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection

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Summary
The pathogen group A Streptococcus (GAS) produces a wide spectrum of infections including necrotizing fasciitis (NF). Streptolysin S (SLS) produces the hallmark β-haemolytic phenotype produced by GAS. The nine-gene GAS locus (sagA–sagI) resembles a bacteriocin biosynthetic operon is necessary and sufficient for SLS production. Using precise, in-frame allelic exchange mutagenesis and single-gene complementation, we show sagA, sagB, sagC, sagD, sagE, sagF and sagG are each individually required for SLS production, and that sagE may further serve an immunity function. Limited site-directed mutagenesis of specific amino acids in the SagA pre-prepropeptide supports the designation of SLS as a bacteriocin-like toxin. No significant pleiotropic effects of sagA deletion were observed on M protein, capsule or cysteine protease production. In a murine model of NF, the SLS-negative M1T1 GAS mutant was markedly diminished in its ability to produce necrotic skin ulcers and spread to the systemic circulation. The SLS toxin impairs phagocytic clearance and promoted epithelial cell cytotoxicity, the latter phenotype being enhanced by the effects of M protein and streptolysin O. We conclude that all genetic components of the sag operon are required for expression of functional SLS, an important virulence factor in the pathogenesis of invasive M1T1 GAS infection.

Introduction
Group A Streptococcus (GAS) is a leading human pathogen causing common infections such as pharyngitis (‘strep throat’) and impetigo (Cunningham, 2000; Bisno et al., 2003). During the last three decades, a resurgence of severe invasive GAS infection has been documented worldwide (Efratiou, 2000). Prominent among invasive GAS syndromes are the destructive soft tissue infection necrotizing fasciitis (NF) and the multisystem disorder of streptococcal toxic shock syndrome (STSS), each carrying significant risk of morbidity and mortality even with aggressive medical therapy (Stevens, 1999; Sharakay et al., 2002). While GAS strains of many M protein (emm) genotypes are capable of producing significant disease, strains representing one globally disseminated clonal M1T1 GAS strain have persisted for over 20 years as the single most prevalent isolate from invasive GAS infections (Cockerill et al., 1997; Cleary et al., 1998; Murono et al., 1999; Chatellier et al., 2000), including all nine surveillance centres of the United States Centers for Disease Control Emerging Infections Program Network in 2002 (http://www.cdc.gov/ncidd/bedmd/abcps).

A hallmark phenotypic feature of GAS is the distinct zone of β-haemolysis surrounding colonies grown on blood agar media. This phenomenon reflects complete lysis of red blood cells produced by the potent oxygen-stable cytolysin known as streptolysin S (SLS). SLS exists primarily in cell-bound form (Ginsburg, 1999) and is delivered most effectively to target cells by direct contact with GAS (Ofeik et al., 1990). The cytolysin spectrum of SLS is broad including lymphocytes, neutrophils, platelets, cancer cell lines and subcellular organelles (Keiser et al., 1964; Taketo and Taketo, 1966; Ginsburg, 1972; Hrynewicz and Pryjma, 1977). Insertion of SLS into the cell membrane results in the formation of transmembrane pores and osmotic cell lysis, similar to that observed with complement-mediated cytotoxicity (Ginsburg, 1999; Carr et al., 2001).

The GAS chromosomal locus for SLS production was first identified by J. DeAzevedo and colleagues through generation and analysis of SLS-deficient transposon

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mutants (Borgia et al., 1997; Betschel et al., 1998). Subsequent chromosome walking studies performed by B. Beall, and informed by the first GAS genome project (Ferretti et al., 2001), recognized the existence of a nine- 
gene cluster (sagA–f for streptolysin-associated genes) in 
the region of transposon insertions. The functional bound-
aries of the sag locus were then defined by plasmid 
integration mutagenesis and its organization as an 
operon confirmed by RNA analysis (Nizet et al., 2000). 
Heterologous expression of the entire GAS sag locus in 
a non-haemolytic strain of Lactococcus lactis yielded 
robust β-haemolytic transformants, confirming that the 
operon is both necessary and sufficient for SLS produc-
tion (Nizet et al., 2000). Individual gene homologies and 
structural features of the operon suggested that SLS is a 
bacteriocin-like peptide toxin, with structural gene sagA 
encoding a 53-amino-acid prepropeptide precursor of the 
mature toxin (Nizet et al., 2000). This hypothesis was 
corroborated by the research group of B. Kreikemeyer (Carr 
et al., 2001), and later others (Dale et al., 2002), who 
demonstrated that antibodies generated against synthetic 
peptides corresponding to the predicted SagA propeptide 
neutralized SLS activity. Highly homologous sag operons 
producing SLS toxins are responsible for the β-haemolytic 
phenotypes of human isolates of groups C and G strep-
tococci (GCS and GGS) and the zoonotic pathogen 
Streptococcus iniae (Fuller et al., 2002; Humar et al., 2002).

Streptolysin S contributes significantly to GAS virulence potential in animal models of infection (Betschel et al., 1998; Humar et al., 2002; Fontaine et al., 2003; Sierig et al., 2003; Engleberg et al., 2004). Like the genetically distinct β-haemolysin of the human pathogen group B Streptococcus (GBS), SLS could theoretically enhance GBS pathogenicity by causing direct tissue injury, promoting 
cellular invasion, impairing phagocytic clearance or 
additional yet to be defined mechanisms (Nizet, 2002). 
However, the analysis of SLS-associated virulence pheno-
notypes has been complicated by reports that various 
mutations in the region of the sagA gene could be 
associated with alterations in expression of other GAS 
virulence genes including M protein, cysteine protease 
and streptokinase (Li et al., 1999; Biswas et al., 2001; 
Mangold et al., 2004).

In the present study, we employ precise in-frame allelic 
replacement, single-gene complementation and limited 
site-directed mutagenesis to test the specific requirement 
of individual GAS sag locus genes for the production of 
SLS. Using a precise allelic replacement, ΔsagA mutant 
created in the background of a serotype M1T1 invasive 
human disease isolate, we then probe the contribution of 
SLS to cellular injury, phagocytic resistance and virulence 
in a murine model of GAS necrotizing soft tissue 
infection.

Results

Requirement of individual sag locus genes in SLS production

Earlier studies establishing the role of the sag locus in 
SLS production employed transposon mutants (Betschel 
et al., 1998; Li et al., 1999) or insertional inactivation 
mutants (Nizet et al., 2000; Biswas et al., 2001) likely to 
evolve polar effects on transcription of downstream genes 
in the operon. To assess the specific requirement of indi-
vidual sag genes in SLS production, we combined precise 
in-frame allelic exchange mutagenesis with single-gene 
complementation analysis. Bacterial strains used in this 
study are listed in Table 1. GAS strain 5448 is a serotype 
M1T1 isolate from a patient with necrotizing fasciitis and 
streptococcal toxic shock syndrome expressing SLS and 
streptolysin O (SLO), cysteine protease, superantigens 
SPEA, SPEF, SPEG and SmeZ and is genetically represen-
tative of the globally disseminated M1T1 clone that is 
the leading cause of invasive GAS infections (Kansal 
et al., 2000). GAS strain NZ131, a serotype M49T14 skin 
isolate from a patient with glomerulonephritis, expresses 
SLS and SLO, and is frequently used in genetic studies 
because of its increased transformability (Simon and 
Ferretti, 1991). Precise in-frame allelic replacement of the 
sagA, sagB, sagC, sagD, sagE, sagF or sagG genes in 
M49 GAS strain NZ131 produced a completely non-
haemolytic phenotype (Fig. 1). Allelic replacement of 
sagA in the M1 GAS strain 5448 also eliminated SLS 
production. Complementation experiments were per-
fomed to reintroduce the corresponding single gene to 
each allelic exchange mutant. Full complementation was 
noted for sagA, sagC and sagF, while partial complement-
lation was seen with sagB, sagD and sagG (Fig. 1). These 
experiments demonstrate a unique requirement for each 
of these six sag operon genes in production of functional 
SLS toxin. We speculate that partial complementation in 
the case of the individual genes mentioned could reflect 
toxicity of overexpression (note SagB and SagD are 
predicted cytoplasmic proteins) or upsetting the stoichiomi-
etry of complex assembly (SagG is predicted to participate 
with SagH and SagI forming an ABC-type transporter).

The sagE gene appears to encode an immunity function

Multiple initial attempts to complement the sagE allelic 
exchange mutant were unsuccessful. Because the pre-
dicted gene product SagE shares homology to a bacteri-
ocin immunity protein of Lactobacillus plantarum, we 
hypothesized that elimination of sagE gene in the face of 
continued SLS biosynthesis may be lethal to the bacte-
rium. We further hypothesized that this may force a com-
mensatory mutation elsewhere in the operon to eliminate 
SLS production allow bacterial survival; hence return of
### Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Group A Streptococcus clinical isolates</strong></td>
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<tr>
<td>NZ131</td>
<td>M49T-14, OF-, emm49 glomerulonephritis isolate</td>
<td>Simon and Ferrell (1991)</td>
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<tr>
<td>s488</td>
<td>M11T, OF-, emm11 necrotizing fasciitis + toxic shock isolate</td>
<td>Kansal et al. (2000)</td>
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<tr>
<td><strong>Group A Streptococcus mutants</strong></td>
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<tr>
<td>NZ131:sagA:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagA by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>NZ131:sagB:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagB by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>NZ131:sagC:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagC by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>NZ131:sagD:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagD by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>NZ131:sagE:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagE by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>NZ131:sagF:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagF by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>This study</td>
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<td>NZ131:sagG:cat</td>
<td>M49 GAS with in-frame allelic replacement of sagG by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
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<td>5448:sagA:cat</td>
<td>M1 GAS with in-frame allelic replacement of sagA by cat (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
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<td>NZ131:Pr-KO</td>
<td>NZ131 emm49 KO by plasmid integration (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
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<td>NZ131:SLO-KO</td>
<td>NZ131 aos KO by plasmid integration (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
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<td>NZ131:sagA::Emr</td>
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<td>NZ131:sagA::MPr-KO</td>
<td>SLS + M protein double KO (Cm&lt;sup&gt;r&lt;/sup&gt; + Em&lt;sup&gt;r&lt;/sup&gt;)</td>
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<td>NZ131:sagA::SLO-KO</td>
<td>SLS + SLO double KO (Cm&lt;sup&gt;r&lt;/sup&gt; + Em&lt;sup&gt;r&lt;/sup&gt;)</td>
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<td><strong>Escherichia coli</strong></td>
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<td>DH1</td>
<td>endA1 hsdR17 (r&lt;sup&gt;m&lt;/sup&gt;15K&lt;sup&gt;r&lt;/sup&gt;) sup E44 thi-1 recA1 gyrA (Nal&lt;sup&gt;R&lt;/sup&gt;) RelA1 lacZ&lt;sup&gt;A1380&lt;/sup&gt;-argF U169, dcm d(lac Z)M15</td>
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<td>MC1061</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; araD199 Δ(lac proAB)lacY1ΔlacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt; F′ lacZΔM15 umc8 Δ(lac proAB)lacY1ΔlacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt; lacY1ΔlacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt; F′ lacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Wernet et al. (1986)</td>
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<td>Top10</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δ(mrr-hsdS-mcrB8&lt;/sup&gt;) F′ lacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt; Δ(lac Z)M15 tcr&lt;sup&gt;r&lt;/sup&gt; Δ(lac proAB)lacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt; F′ lacZΔM15 tcr&lt;sup&gt;r&lt;/sup&gt;</td>
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<td><strong>Lactococcus lactis</strong></td>
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<td>NZ9000</td>
<td>MG1363 (lacking nisin operon); papN::nisRIK</td>
<td>Kuipers et al. (1998)</td>
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<td><strong>Plasmid</strong></td>
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<td>pCR2.1-TOP1</td>
<td>ColE1 ori, Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, lacZ&lt;sup&gt;A&lt;/sup&gt;, T-A cloning vector</td>
<td>Invitrogen</td>
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<td>pHY304</td>
<td>Temp&lt;sup&gt;R&lt;/sup&gt; pVE6007α derivative, Em&lt;sup&gt;R&lt;/sup&gt; + lacZ&lt;sup&gt;A&lt;/sup&gt;MSC of pBlueScript</td>
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<td>pACYC184</td>
<td>rep(p15A), Cm&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Rose (1988)</td>
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<td>pVE6007α</td>
<td>Temperature sensitive derivative of pWV01, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pSagB::cat-KO</td>
<td>pHY304 containing sagB::cat allele + flanking DNA, Em&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td><strong>Complementation and heterologous expression studies</strong></td>
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<td>pDC125</td>
<td>E. coli streptococcal promotorless shuttle vector, JS-3 replicon, Em&lt;sup&gt;R&lt;/sup&gt; (erm)</td>
<td>Chaffin et al. (1998)</td>
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<td>pDC123</td>
<td>E. coli streptococcal shuttle expression vector, JS-3 replicon, Cm&lt;sup&gt;R&lt;/sup&gt; (cat)</td>
<td>Chaffin and Rubens (1998)</td>
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<td>pDCerm</td>
<td>pDC123 derivative with Em&lt;sup&gt;R&lt;/sup&gt; (erm of Tn9166E) replacing cat</td>
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<td>pADsagA</td>
<td>pDC125 + NZ131 sagA gene + the native sag promoter</td>
<td>This study</td>
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<td>pADsagB</td>
<td>pDCerm + NZ131 sagB gene behind vector promotion</td>
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<td>pADsagC</td>
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<td>pADsagA with site-directed mutation of proline 21 to alanine</td>
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<td>pADsagG23A</td>
<td>pADsagA with site-directed mutation of glycine 23 to alanine</td>
<td>This study</td>
</tr>
<tr>
<td>pADsagG24A</td>
<td>pADsagA with site-directed mutation of cysteine 24 to alanine</td>
<td>This study</td>
</tr>
<tr>
<td>pADsagC27A</td>
<td>pADsagA with site-directed mutation of cysteine 27 to alanine</td>
<td>This study</td>
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<tr>
<td>pADsagK53A</td>
<td>pADsagA with site-directed mutation of lysine 53 to alanine</td>
<td>This study</td>
</tr>
<tr>
<td>pSagLocus</td>
<td>pDC123 + 9440 bp PCR amplicon of entire nine-gene sag operon</td>
<td>Nizet et al. (2000)</td>
</tr>
<tr>
<td>pSagLucus::Emr</td>
<td>pSagLocus with SagE deleted leaving SagF ribosome binding site intact</td>
<td>This study</td>
</tr>
<tr>
<td>pDL278</td>
<td>E. coli streptococcal shuttle vector, pYA306-1 replicon, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>LeBlanc et al. (1992)</td>
</tr>
<tr>
<td>pDL278-sagE</td>
<td>pDL278 + NZ131 sagE gene behind vector promotion</td>
<td>This study</td>
</tr>
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</table>
sagE alone in trans could not restore the SLS phenotype. To address this issue experimentally, we performed allelic exchange mutagenesis of sagE in the chromosome after pre-transforming GAS with a vector expressing sagE. Allelic replacement of sagE with cat yielded SLS-positive GAS when the chromosomal gene substitution was performed in bacteria harbouring a second copy of sagE in trans throughout (Fig. 1). These results strongly suggest an immunity function encoded by sagE. It remains unproven whether the sagE gene itself is also required for SLS biosynthesis. Also, it appears that sagE-mediated immunity is related only to the endogenous production of SLS and not exogenous exposure to SLS. Colonies of the sagE allelic exchange mutant could grow normally within a zone of β-haemolysis surrounding a wild-type colony, and equivalent growth of wild-type and sagE mutant GAS occurred in mixed liquid culture (data not shown).

Heterologous expression of individual sag genes

The complete sag operon cloned in an expression vector (pSagLocus) can confer SLS production to Lactococcus lactis. When the complementation vectors produced in this study were used to transform L. lactis, no single gene from sagA to sagG was sufficient for SLS production (not shown). A construct harbouring a deletion of sagE alone from plasmid pSagLocus (pSagLocusΔE) also failed to produce SLS when expressed in L. lactis, suggesting the role of SagE in immunity and/or production of SLS is also required for heterologous expression.

Site-directed mutagenesis of the SagA propeptide

Several structural features of the GAS sag operon suggest that SLS is related to the bacteriocin family of small peptide toxins (Nizet et al., 2000; Ferretti et al., 2001; Wescombe and Tagg, 2003). The structural gene sagA encodes a 53-amino-acid product (Fig. 2) containing a predicted glycine–glycine cleavage characteristic of non-lanthionine-containing bacteriocins of Gram-positive bacteria (Jack et al., 1995) as well as certain lantibiotics such as salivaricin A (Ross et al., 1993). Cleavage at this site would remove a 23-amino-acid leader peptide from a mature 30 SLS propeptide matching the predicted size.
of SLS (2.9 kDa) from earlier biochemical analyses (Koyama, 1963; Bemheimer, 1967). The SagA sequence also shares a proline at the ~3 position from the cleavage site with the leader peptide of microcin B17 of Escherichia coli (Madison et al., 1997). We found that mutation of this SagA proline (21) to alanine led to complete loss, and mutation of SagA glycine (23) to alanine led to a significant decrease in the haemolytic activity of GAS on SBA (Fig. 2). High cysteine content is a common feature of bacteriocin propeptides, and these residues can link to dehydrated serine and threonine residues (in lantibiotics), to dehydrated glycine residues (as in Microcin B17), or to one another (as in the cysbacteriocins pediocin PA-1 and leuconic A) (Jack et al., 1995). We found changing either cysteine (24) or cysteine (27) to alanine led to complete abolishment of SLS activity (Fig. 2). Finally, the very C-terminal residue of several lantibiotic (e.g. nisin, subtilin) and non-lantibiotic (e.g. sakacin, lactacin) bacteriocin propeptides is lysine as in SagA. Mutation of lysine (53) to alanine eliminated SLS haemolytic activity (Fig. 2). Our limited analysis of SagA by site-directed mutagenesis of conserved residues supports its assignment as bacteriocin-like peptide toxin.

**Elimination of sagA does not produce significant pleiotropic effects**

In standard Todd–Hewitt broth (THB) media, the seven individual M49 mutants in sagA–sagG genes grew equally well as parent strain NZ131, as did allelic exchange mutant 5448:sagA3:cat compared with M1 parent strain 5488. An earlier study of a transposon mutant disrupting sagA associated the mutation with loss of SLS expression but also with decreased expression of the genes encoding M protein and cysteine protease SpeB (Li et al., 1999). Another study using an insertion duplication mutation of sagA associated the mutation with loss of SLS expression and with decreased surface expression of the M protein (Biswas et al., 2001). Our precise in-frame allelic exchange mutant of sagA in the M1 background is free of polar effects the downstream operon (as verified by single-gene complementation), and did not show differences in emm gene transcription nor M protein expression as determined by reverse transcription polymerase chain reaction (RT-PCR) (Table 2) and whole-cell dot blot analysis (Fig. 3A) respectively. Removal of sagA also did not affect transcription of the speA gene encoding the GAS pyrogenic exotoxin A (Table 2). The SagA mutant had slightly increased levels (fourfold) of mRNA production for the speB gene (Table 2) and immunodetectable SpeB protein (Fig. 3A), but these differences did not translate into a significant increase in the amount of cysteine protease activity exhibited by the whole bacterium (Fig. 3B). Finally, procedures for genetic manipulation of GAS may inadvertently select mutants with decreased expression of the surface hyaluronic acid capsule, leading to potential misinterpretation of experimental results (Ashbaugh et al., 1998). We found that sagA mutagenesis did not affect GAS expression of hyaluronic acid capsule (Fig. 3C). The lack of pleiotropic effects on M protein and capsule are
Table 2. Real-time RT-PCR analysis of gene expression in wild-type and ΔsagA mutant GAS.

<table>
<thead>
<tr>
<th>GAS strain</th>
<th>RecA</th>
<th>SpeA</th>
<th>M protein</th>
<th>SpeB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of molecules (×10^6)</td>
<td>No. of molecules (×10^6)</td>
<td>Normalized ratio</td>
<td>No. of molecules (×10^6)</td>
</tr>
<tr>
<td>Wild-type 5448 (M1)</td>
<td>11.1</td>
<td>5.76</td>
<td>0.0053</td>
<td>14.75</td>
</tr>
<tr>
<td>SLS– mutant</td>
<td>12</td>
<td>6.13</td>
<td>0.0050</td>
<td>14.1</td>
</tr>
<tr>
<td>S448ΔsagAΔcat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

especially important as each is known to contribute to GAS tissue necrosis and virulence in the murine model (Ashbaugh et al., 1998).

SLS is important for the pathogenesis of M1 GAS necrotizing soft tissue infection

M1 strains are the most common isolates associated with the recent resurgence of NF and other invasive GAS infections. To determine the importance of SLS for disease progression, we injected mice subcutaneously with 10^7 colony-forming units (cfu) of wild-type strain M1 GAS 5448 or its isogenic SLS-negative mutant 5448ΔsagAΔcat. Six of eight mice infected with the wild-type strain developed large necrotic ulcers beginning between 24 and 48 h, while only one of eight SLS-negative mutant ulcer, itself very small (Fig. 4A). Mice were sacrificed at 96 h with collection of lesion (or inoculation site) biopsies and blood for quantitative culture. Consistent with larger lesions (P < 0.002), the wild-type GAS strain was present in much higher concentrations in the skin (mean cfu g^-1 = 1 × 10^9) than the SLS-negative mutant (mean cfu g^-1 = 4 × 10^6) (P < 0.001) (Fig. 4B). Seven of eight mice infected with the wild-type strain were bacteremic at 96 h (mean cfu ml^-1 = 2 × 10^8) compared with three of eight mice infected with the SLS-negative mutant (mean cfu ml^-1 = 1 × 10^6) (P < 0.001). Histopathologic studies were performed on excised skin and subcutaneous tissue with representative light micrographs shown in Fig. 5. Extensive necrosis of the skin and subcutaneous fatty tissues was seen in the six mice infected with wild-type M1 GAS that developed ulcers and thrombosis of bridging blood vessels, with abundant bacteria and dense neutrophilic infiltrates throughout the ulcer margin (Fig. 5B and C). In two of these mice, deeper involvement with myonecrosis was seen (Fig. 5D). In contrast, mice infected with the SLS-negative mutants had minimal inflammation without necrosis and mild perivascular neutrophilic infiltrates (Fig. 5E and F), findings that resembled the histopathology of mock-infected mice injected with sterile Cytodex beads alone (Fig. 5A). The in vivo studies demonstrate that SLS is an important virulence factor in M1 GAS skin and soft tissue infection, contributing to tissue injury and promoting systemic spread of the bacterium.

SLS does not contribute to epithelial cell invasion or cathelicidin resistance

It has been proposed that the increased virulence of M1 GAS strains is associated with a capacity for high frequency intracellular invasion of human epithelial cells (LaPenta et al., 1994; Cleary et al., 1998). We found that

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Fig. 3. Analysis of other virulence phenotypes in wild-type and sagA mutant GAS. A. Immunoblot analyses for cysteine proteinase SpeB and M protein. B. Assay for cysteine proteinase enzymatic activity. C. ELISA for hyaluronic acid capsule production.
the cathelicidin class of cationic anti-microbial peptides (Dorschner et al., 2001; Nizet et al., 2001). We found no difference in the susceptibility of the M1 wild-type strain and its isogenic ΔsagA mutant to the murine cathelicidin mCRAMP (minimum inhibitory concentration (MIC) = 16 μM for both). Likewise, no difference in cathelicidin susceptibility was observed when comparing M49 parent strain NZ131 to its isogenic ΔsagA, ΔsagB, ΔsagC, ΔsagD, ΔsagE, ΔsagF and ΔsagG mutants (not shown).

**SLS promotes resistance to phagocytic killing**

One mechanism by which SLS expression could contribute to bacterial proliferation in vivo is by impairing host phagocytic clearance mechanisms. Killing assays were performed on M1 and M49 wild-type and sagA allelic exchange mutant GAS using fresh human blood from several donors. While the wild-type strains proliferated in human blood over the 2 h assay period, the SLS-negative mutants were effectively cleared (Fig. 6A). Additional studies were performed with purified human neutrophils using M1 GAS opsonized in 10% autologous serum. Again, the wild-type GAS strain proliferated to a much greater extent than the SLS-negative mutant strain in the neutrophil bactericidal assay (Fig. 6B). These results indicate that SLS is an important component of GAS resistance to phagocytic clearance.

**SLS contributes to direct host cell injury**

Partially purified preparations of SLS have been reported to show potent cytolytic activity against a broad range of eukaryotic cell membranes. To determine the contribution of SLS to cytotoxicity in the context of GAS–epithelial cell interaction, we exposed the human keratinocyte cell line HaCat to wild-type and SLS mutant GAS and assessed cell death by trypan blue nuclear staining. Figure 7A shows that the SLS- mutant was associated with drastically reduced epithelial cell killing compared with wild-type (6% versus 72%), while restoration of SLS activity to the mutant with a sagA complementation vector restored the wild-type cytolytic phenotype. Representative Trypan blue-stained epithelial monolayers from these experiments are seen in Fig. 7B. We hypothesized that the M protein of GAS may facilitate close bacterial–epithelial cell contact that would enhance SLS-mediated cytotoxicity. Figure 7C shows experiments performed with A549 human lung epithelial monolayers exposed to wild-type GAS and isogenic single and double mutants in SLS and M protein in which cell injury was measured by lactate dehydrogenase (LDH) release. We found that both SLS and M protein contributed significantly to GAS cytotoxicity (~50% reduction in LDH release in single mutants), and that a further reduction (~70% reduction from wild type)
was seen in a mutant lacking both virulence determinants. In a parallel experiment using isogenic single and double mutants of SLS and the GAS pore-forming cytolysin SLO, we found that SLS was a more important contributor to GAS cytolytic action, and that a mutant lacking both toxins had a further reduction of cytolyis to very low levels (Fig. 7C). Note that these experiments were performed at atmospheric oxygen concentrations with 5% CO₂, and SLO is known to be oxygen labile.

**Discussion**

Although β-haemolysis is often applied synonymously to the GAS bacterium, the SLS toxin responsible for this signature phenotype remains enigmatic and incompletely understood. Earlier work has shown that the nine-gene GAS operon sagA–sagI is necessary and sufficient for SLS biosynthesis (Betschel et al., 1998; Niset et al., 2000), but the use of transposon or plasmid integrational mutagenesis techniques with potential for polar mutations in these studies failed to provide definitive proof of the role of individual sag genes in toxin production. Here we use precise, in-frame allelic replacement and corresponding single-gene complementation to demonstrate that the sagA, sagB, sagC, sagD, sagF, and sagG genes are specifically required for SLS production, and to provide strong suggestive evidence that the sagE gene encodes an immunity function against SLS autotoxicity.

The sag operon has many features reminiscent of a biosynthetic apparatus for a bacteriocin-like small peptide toxin. By sequence homology, these features include a probable prepropeptide structural gene (sagA) and ATP-binding cassette-type export apparatus (sagG–I), and with considerably less certainty, genes encoding a candidate bacteriocin modifying enzyme (sagB) and bacteriocin immunity protein (sagE) (Niset et al., 2000). Without significant GenBank homologies to genes of known function, sagC and sagF are predicted to be membrane proteins, and sagD a cytoplasmic protein, by the PSORT localization algorithm (Nakai and Horton, 1999). Our allelic replacement and single-gene complementation of sagC, sagD and sagF confirm the requirement of these individual genes for SLS production, but their precise functions in the toxin biosynthetic or export process

![Fig. 5. Histopathology of skin and subcutaneous tissues at 96 h post challenge with M1T1 GAS.](image)

A. Normal histopathology seen in a mock-infected animal.

B and C. In animals challenged with wild-type GAS there is evidence of necrotic ulcer formation with thrombosis (B) and under higher power neutrophilic inflammatory infiltrates (C).

D. Myonecrosis was seen in two animals.

E, F Mice challenged with the saga mutant did not develop skin necrosis and had only mild perivascular inflammatory cell infiltrates.

![Fig. 6. Anti-phagocytic properties of streptolysin S.](image)

A. Relative survival in human whole blood of wild-type and saga mutant GAS, using eight different donors. Growth index = surviving cfu divided by cfu in the input inoculum.

B. Similar comparison in an assay using purified human neutrophils and autologous serum.
remain undefined. Preparations of stabilized SLS are lytic to bacterial protoplasts and spheroplasts (Bernheimer, 1966), but to date no definitive bacteriocidal activities against cell-wall competent bacteria have been defined in co-culture experiments comparing wild type and SLS-negative mutant GAS (Nizet et al., 2000).

The identity of SagA as the prepropeptide precursor of SLS was strongly suggested by the predicted small size of mature SLS (Koyama, 1963; Bernheimer, 1967; Alouf and Loridan, 1988) and confirmed through neutralization of SLS activity with antibodies directed against synthetic peptides representing SagA C-terminal sequences (Carr et al., 2001; Dale et al., 2002). Our allelic replacement and single-gene complementation of sagA corroborated its absolute necessity for SLS production, and provided a platform for testing the requirement of several amino acid residues characteristic of the bacteriocin toxin family by plasmid-based site-directed mutagenesis. The predicted processing site of the SLS prepeptide is preceded by the sequence Pro–Gly–Gly. Pro(21) and the Gly(23) are conserved with residues in the leader peptide required for the post-translational processing of the McbA prepropeptide precursor of Microcin B17 (Madison et al., 1997). We found that mutation of SagA Pro(21) to Ala eliminated SLS activity while mutation of Gly(23) to Ala led to significantly diminished, but not absent, β-haemolysis on blood agar. We hypothesize that the Gly to Ala substitution is sufficiently conservative to allow partial recognition by the SLS leader peptidase, and indeed Gly–Ala processing sequences are found in a small proportion of bacteriocin precursors including the lantibiotic SA-FF22 expressed by certain GAS strains (Jack et al., 1994).

The unusual initial string of five Cys residues at the start of the predicted prepeptide sequence (six in the S. iniae
SLS homologue) was targeted by replacing Cys(24) or Cys(27) with alanine, each time eliminating SLS activity. This result suggests that thioether bond formation characteristic of the post-translational modification of other bacteriocins may be an important step in the generation of mature SLS. Future biochemical analyses beyond the expertise of our laboratory will identify whether the target residues for cyclization are the abundant Thr (29, 30, 36, 50) or Ser (34, 39, 42, 46, 48) residues that could undergo dehydration to form thioether bonds seen in lantibiotics (Sahl and Bierbaum, 1998), or the many glycine residues (38, 40, 44, 45, 47, 52) that could potentially participate in formation of thiazole or oxazole rings reminiscent of Microcin B17 (Madison et al., 1997). Our site-directed mutagenesis also indicates the Lys(53) residue is essential for SLS toxin processing or activity. An immediate C-terminal Lys is also present in the lantibiotic bacteriocin nisin, and chemical blockage of that Lys results in marked reduction of the toxin’s pore-forming potential (van Kraaij et al., 1998).

Allelic replacement and single-gene complementation prove the requirement of two additional genes for SLS production: the sagB gene encoding a candidate bacteriocin modifying enzyme and the sagG gene encoding a protein with the signature ATP binding pocket motifs of an ABC-type transporter. These results are consistent with the processing and export pathway of a bacteriocin-type toxin. The predicted protein product of the sagA gene shares weak homology with a candidate immunity protein PinP of Lactobacillus plantarum (Diep et al., 1996). Our discovery that allelic replacement of sagE and single-gene complementation to restore SLS activity occurs only if a copy of sagE is introduced in trans before completing the chromosomal deletion is consistent with its gene product serving an immunity function. Little is known about the precise mechanisms of bacteriocin immunity; however, a concept based on target shielding has been recently proposed for the bacteriocin immunity protein Pepl1 of Staphylococcus epidermidis in which a hydrophobic N-terminal domain (24 of 26 non-polar residues) and a hydrophilic and net-positively charged C-terminus (NSNKDKDL) are the essential components (Hoffmann et al., 2004). Curiously, the N-terminus of GAS SagE has no charged amino acids among its first 28 residues, and its C-terminus also ends in a hydrophilic and net-positively charged sequence (TKKKKEVT).

In our experiments with the M1T1 GAS parent strain, we did not observe strong pleiotropic effects of sagA deletion on expression of M protein, cysteine protease SpeB, pyrogenic exotoxin A or hyaluronic acid capsule. These findings are consistent with earlier analysis of transposon insertion mutants in the sag promoter region of M1 and M18 strains (Betschel et al., 1998) and a non-polar deletion mutant of sagB in an M5 GAS strain (Fontaine et al., 2003). Separate investigations have attributed global regulatory functions to the sagA gene itself, applying the designation pel, for ‘pleiotropic effects locus’, to the open reading frame (ORF). A M49 serotype Tn917 ‘pel’ mutant with an insertion in the region of the sag promoter was non-haemolytic and showed decreased transcription of the genes for M-protein, SpeB and streptokinase (Li et al., 1999). Passage of this mutant in mice selected for restoration of sagA transcription and β-haemolysis but did not reverse all of the pleiotropic effects (Eberhard et al., 2001). In contrast, an insertion duplication mutation of the sagA gene in an M6 GAS strain was shown to have normal emm transcription but to express a truncated version of the M-protein that would not anchor to the cell surface (Biswas et al., 2001). A recent well-designed study provides compelling evidence that the untranslated RNA sequence comprising sagA can act as a growth phase-dependent positive regulator of the emm and sic genes at the transcriptional level and of SpeB at the post-translational level (Mangold et al., 2004). Further complexity is presented by the fact that the expression level of the sag locus is under transcriptional control of the GAS global regulators CovR/CovS (also known as cfrR/cfrS), rolA, mga and fas (Kihlberg et al., 1995; Federle et al., 1999; Heath et al., 1999). In the present post-genomic era of GAS research, new studies to analyse the complete transcriptome of wild type and ΔsagA mutants under a variety of in vitro and in vivo conditions will be helpful in elucidating the intriguing regulatory roles of the locus.

We found SLS expression to be critical for the pathogenesis of M1T1 GAS infection in a murine model of necrotizing soft tissue infection, corroborating previous studies indicating a virulence role of SLS in lesion size or mortality in GAS, GGS and S. iniae skin infection (Betschel et al., 1998; Fuller et al., 2002; Humar et al., 2002; Fontaine et al., 2003; Engleberg et al., 2004). We further found that SLS expression was strongly correlated to viable bacterial counts in the infected skin tissue and to the ability of the organism to rapidly disseminate and produce high-grade bacteraemia. Histopathological examination at day 4 showed severe necrosis, widespread bacterial infiltration and abundant neutrophil infiltration in the ulcer tissue of most animals infected with wild-type M1 GAS. In contrast, minimal evidence of necrosis and tissue injury was seen in mice challenged with the SLS mutant. The necrotic tissue damage is likely to reflect a potent direct cytolytic effect of SLS on host cell types resident in the skin and subcutaneous tissues. This finding was confirmed in our in vitro essays in which SLS was seen to produce direct cytolytic injury to human keratinocytes. We found that SLS may act synergistically with the oxygen-labile GAS exotoxin SLO to effect host cell injury, and that SLS cytotoxicity was also enhanced by the presence of M
protein, whose adhesin properties could facilitate the close interaction between the bacteria and target cell membrane required for optimal SLS delivery (Ofek et al., 1990; Ginsburg, 1999).

Compared with the SLS mutant, several log-fold greater quantities of viable wild-type GAS were present in the infected tissue biopsies despite the presence of markedly increased numbers of neutrophils recruited to the site of infection. This potential paradox suggested that SLS must contribute in some fashion to resistance against phagocytic clearance mechanisms. Indeed our studies confirmed this to be the case as the ΔsagA mutant did not survive as well as wild-type GAS in killing assays with human whole blood and purified human neutrophils. These results contrast with recent observations for whole blood survival of an SLS-deficient sagB mutant of M5 GAS (Fontaine et al., 2003) or neutrophil resistance of an SLS-deficient M3 strain of GAS (Siering et al., 2003) in which negligible differences were seen in comparison to the parent strains. Neutralizing antibodies against the SagA propeptide sequence were found to enhance neutrophil opsonophagocytosis of an M24 strain of GAS (Dale et al., 2002). Thus it is apparent that strain differences may exist in the relative contribution of SLS to phagocyte resistance, and that other well-studied phenotypic characteristics such as the M protein or hyaluronic acid capsule may play a more prominent role in certain backgrounds. In our animal studies, a role of SLS in phagocytic resistance may facilitate bacterial proliferation in the face of a brisk neutrophilic inflammatory response elicited in response to the toxin-mediated tissue injury. Consequently, both SLS cytotoxicity- and neutrophil-mediated inflammatory damage may contribute to the observed necrotic ulcer formation.

In sum, we have used a molecular genetic approach to study the unique requirement of individual sag locus genes to SLS production and the role it plays in the pathogenesis of GAS M1T1 necrotizing skin and soft tissue infection. We hope the insights and reagents we have generated can prove useful for future studies of the biology and biochemistry of this unusual bacteriocin-like toxin.

Experimental procedures

Bacterial culture and transformation conditions

GAS were grown in THB, on Todd–Hayfitt agar plates (THA) or on plates of trypticase soy agar + 5% sheep red blood cells (SBA). For antibiotic selection, 2 μg ml⁻¹ erythromycin (Em), 1 μg ml⁻¹ chloramphenicol (Cm) or 500 μg ml⁻¹ spectinomycin (Sp) was added to the media. To prepare log-phase GAS for use in tissue culture assays or animal infections, bacteria were grown to OD₆₀₀ = 0.4 to 1.0 cfu ml⁻¹, pelleted, washed, resuspended and diluted in PBS or tissue culture media to the desired concentration. E. coli strains were grown in Luria–Bertani broth or on Luria–Bertani agar plates; antibiotic selection utilized 100 μg of ampicillin (Amp) per ml, 500 μg of Em per ml, or 5 μg of Cm per ml. L. lactis was grown in M17 broth (Difco) supplemented with 1% glucose (GM17) or on GM17 agar plates with selection of Cm or Em at 5 μg ml⁻¹. GAS were rendered transformable by electroporation through growth in THB + 0.3% glycine (M49) or THB + 1.25% glycine + 0.5 M glucose + 5% sucrose (M1), then prepared as described for Streptococcus agalactiae (Franson et al., 1997). L. lactis was made transformable by growth in GM17 plus 2.5% glycine (Holo and Nes, 1989). After electroporation (Eppendorf 2510, 1.5 kilovolts), cells were incubated in THB + 0.25 M sucrose (GAS) or GM17 media + 20 mM MgCl₂ + 2 mM CaCl₂ (L. lactis) for 1–2 h before antibiotic selection on agar media.

Precise in-frame allelic exchange mutagenesis

PCR was used to amplify NZ131 chromosomal DNA fragments containing sagA, sagB, sagC, sagD, sagE, sagF or sagG with several hundred base pairs upstream and downstream sequence in each case. The primer sequences employed have been published (Nizet et al., 2000) and were paired as follows: sagAUpFw + sagARev to amplify the sagA gene within a 1648 bp ampiclon, sagAFwd + sagCRev to amplify the sagB gene within a 2250 bp ampiclon, sagBFw + sagDRev to amplify the sagC gene within a 2556 bp ampiclon, sagCFw + sagERev to amplify the sagD gene within a 2146 bp ampiclon, sagDFw + sagFRev to amplify the sagE gene within a 2068 bp ampiclon, sagEFw + sagGRev to amplify the sagF gene within a 1641 bp ampiclon, and sagFFwd + sagHRev to amplify the sagG gene within a 1925 bp ampiclon. Each PCR product was T-A cloned in pCR2.1 (Invitrogen). These vectors (pSagA-TV, pSagB-TV, etc.) served as templates for inverse PCR reactions using (i) a reverse primer immediately upstream of the stop codon and (ii) a forward primer immediately after the stop codon of the cloned ORFs; these primers were designed with 25 bp 5'-extensions corresponding to the start and end of the chloramphenicol acetyltransferase gene (cat) respectively. The resultant linearized PCR products, containing an in-frame deletion of the individual sag gene, were used to transform E. coli Top10 together with an ~650 bp PCR ampli-

con of the complete cat gene from pACYC184. In vivo recombination events were identified by screening for Top10 exhibiting AmpR + CmR, and verified by PCR and restriction analysis to contain an in-frame substitution of the target gene(s) with cat. The mutated sagA, sagB, sagC, sagD, sagE, sagF or sagG in the M49 chromosome and sagA in the M3 chromosome were confirmed by (i) PCR using cat primers with upstream and downstream
prismers and (ii) absence of amplification of the wild-type gene.

**Single-gene complementation analysis**

To reintroduce *saga* and its native promotor in trans to the M1 and M49 GAS allelic exchange mutants 5448:sagaA::cat and NZ131:sagaA::cat, the PCR amplicon from pSagA-TV was subcloned as a BamHI + XbaI fragment into pDC125 (Chaffin et al., 1998), a streptococcal/E. coli shuttle vector designed with transcriptional terminators to prevent promotion from plasmid backbone. The resultant complementation vector pADsagaA was introduced into 5448:sagaA::cat and NZ131:sagaA::cat by electroporation, transformants identified by growth on THA + Cm + Em, and plasmid integrity confirmed by restriction and PCR analysis. To reintroduce individual downstream genes of the *sag* operon in trans to their corresponding *cat* allelic exchange mutants, we used plasmid pDCerm (Jeng et al., 2003), an Em<sup>+</sup> streptococcal/E. coli shuttle vector that we derived from pDC123 (Chaffin and Rubens, 1996). This vector contains constitutive promoters on the plasmid backbone to allow expression of the cloned gene. The PCR amplicons of each downstream *sag* gene were recovered from vectors pSagB-TV, pSagC-TV, etc. by digestion with BamHI + XbaI and subcloned to pDCerm prepared with the compatible restriction enzymes. The single-gene complementation vectors pADsagaB, pADsagaC, etc. were introduced to the corresponding GAS M49 allelic exchange mutants by electroporation, transformants identified by growth on THA + Cm + Em, and plasmid integrity confirmed by restriction and PCR analysis. Complemented mutants were screened for SLS activity by growth on SBA with appropriate antibiotic selection.

**Allelic exchange mutagenesis of sag E**

The *sagE* gene and flanking DNA from pSagE-TV was cloned into expression vector pDL278 (LeBlanc et al., 1992) to yield pDL278-sagE. GAS NZ131 was transformed with pSagE::cat.KO as above, temperature shift performed, and a non-haemolytic, downstream single-crossover integration within the *sagE* ORF confirmed by PCR and Cm<sup>+</sup> + Em<sup>+</sup>. This single cross-over mutant was rendered electrocompetent and transformed with either pDL278 or pDL278-sagE with selection of Sp<sup>c</sup> colonies. While maintaining Sp<sup>c</sup> selection, the temperature was relaid to 30°C for several passages, then plated at 37°C for identification Cm<sup>+</sup> but Em<sup>+</sup> colonies. Precise allelic replacement of SagE with *cat* in the chromosome was confirmed by PCR.

**Site-directed mutagenesis of SagA**

PCR-based site-directed mutagenesis (ExSite<sup>TM</sup>, Stratagene) was performed on selected amino acids within the SagA encoding sequence within complementation vector pAD-sag4. Primers were designed to replace the targeted codon with a codon for alanine. DpnII was used to remove template DNA before transformation and recovery of mutated plasmids in *E. coli* MC1061. The fidelity of the site-directed mutation was confirmed by direct sequence analysis, and the modified plasmids were used to transform NZ131:sagaA::cat for assessment of SLS phenotype.

**Real-time RT-PCR analysis**

RNA was extracted using the RNeasy kit (Qiagen) from mid-log-phase GAS lysed mechanically with a FastPrep instrument (Bio 101). The samples were treated with DNase and the quality of RNA was verified spectrophotometrically. The total RNA isolated from the bacterial isolates as well as an RNA standard for each gene of interest (emm1, speA, speB) were converted to cDNA using random hexamers. To confirm the absence of DNA template, conventional PCR was used with or without reverse transcriptase. Gene expression was quantified using an ABI PRISM 7900HT® sequence detection system using gene-specific primers in PCR Master Mix (Applied Biosystems, Foster City, CA) containing SYBR Green. RNA level of the housekeeping gene *recA* was also quantified. The data from the real-time PCR reaction was generated at CT values (threshold cycle at which there was a significant increase in signal generated by any given set of PCR conditions), which were then converted to number of RNA molecules using the gene-specific standard curves. These values were divided by the calculated number of housekeeping gene RNA molecules to allow for normalization of intra- and interexperimental data.

**Phenotypic analysis of GAS virulence factors**

The amount of mature SpeB secreted by each isolate and migrating at 20 kDa was determined by Western immunoblots developed with anti-SpeB antibodies as described (Kansal et al., 2000). The proteolytic activity of SpeB was detected using the EnzCheck protease assay kit (Molecular Probes) as described in detail in Kansal et al. (2000). M protein dot blot analysis on whole GAS cells was performed with antibodies to the N-terminal domain of M protein as described (Kansal et al., 2000). ELISA for hyaluronic acid capsule was performed using the HA test kit (Corigenex) per manufacturer's instructions. Minimum inhibitory concentrations for murine cathelicidin mCRAMP against GAS strains were determined as described previously (Nizet et al., 2001).

**Murine model of GAS necrotizing skin infection**

Experiments were performed using a well-established model (Betschel et al., 1998; Nizet et al., 2001; Humar et al., 2002). Briefly, GAS were grown to log phase, pelleted, washed, resuspended in PBS, and mixed 1:1 with Cytodex beads (50 mg per 100 ml, Sigma). An inoculum of 10<sup>7</sup> cfu GAS in 0.2 ml was then injected into the right flank of 4-week-old male hairless cfr:SKH1/hr/hr Br mice (n = 8 per group). Animals were monitored daily for development of necrotic ulcers and weight loss. At 96 h, all animals were sacrificed, biopsies performed for histopathologic assessment (H&E staining), and skin, blood and spleen collected for quantitative culture.
Whole blood and neutrophil killing assays

For blood killing, the method of de Malmanche and Martin (1994) was employed. Briefly, GAS were grown to log phase and diluted in PBS to 10^6 cfu in 100 ml, then mixed with 300 ml of fresh heparinized (10 U ml⁻¹) human whole blood in siliconized tubes. The mixture was incubated at 37°C for 2 h with orbital shaking, then plated on agar media for enumeration of surviving cfu. Growth index is defined as the number of surviving cfu divided by the input inoculum. Experiments were repeated a total of 10 times using six different donors. Human neutrophils were purified using the PolymorphPrep™ (Grenier Bio-One) per manufacturer’s instructions, then used in a GAS bacterial assay precisely as described by Kobayashi et al. (2003).

Mutagenesis of M protein and SLO genes

PCR was used to amplify internal fragments of the emm49 gene (900 bp from codon 17 to 258) and slo gene (1151 bp from codon 78 to 468) from NZ131. These fragments were cloned into temperature-sensitive suicide vector pVE60073, to yield targeting vectors pVEemm49-KO and pVESlo-KO respectively. These vectors were used to transform wild-type NZ131 and the sAG allelic exchange mutant NZ131.sAG:Emr (Nizet et al., 2000) at the permissive temperature (30°C). Campbell-type plasmid integrational mutagenesis of emm49 or slo was then achieved by shift to the non-permissive temperature (37°C) while maintaining Cm selection. PCR was used to confirm the targeted disruption of the genes yielding the single mutants NZ131.M-Pr.KO and NZ131:SLO.KO and the double mutants NZ131.sAG:A-M-Pr.KO (SLS + M protein) and NZ131.sAG:SLO.KO (SLS + SLO).

Cytotoxicity and cellular invasion assays

The human keratinocyte cell line HaCat (Boukamp et al., 1988) was propagated in RPMI media (Gibcö-BRL) supplemented with 10% fetal bovine serum and seeded in four-well chamber slides (Microtek). Newly confluent monolayers were exposed to log-phase GAS strains at multiplicity of infection (moi) of 1000:1 and incubated for 2 h at 37°C in 5% CO₂. Excess bacteria were removed by gentle washing in PBS, monolayers stained with 0.04% trypan blue, then fixed with glutaraldehyde and counterstained with eosin for quantification of stained nuclei under light microscopy as described (Gibson et al., 1999). The human lung epithelial cell line A549 (ATCC #CCL-185) was propagated in RPMI media supplemented with 10% FCS and seeded in 24-well tissue culture plates (Corning). For cytotoxicity assays, log-phase GAS were added at 10⁵ cfu per well (moi = 100:1), spun at 200 g to place bacteria on the monolayer surface, and incubated for 2 h at 37°C in 5% CO₂. LDH release was determined using a miniaturized version of the Sigma colorimetric assay (catalogue #500-C) as described (Nizet et al., 1996). LDH assays were performed in quadruplicate and repeated for three times. Assays for GAS intracellular invasion of A549 cells were performed as previously described for S.agalactiae (Doran et al., 2002) using moi = 1:1 for 2 h.

Statistical analyses

Data sets were compared by Student’s t-Test using the Microsoft Excel statistical package; a P-value <0.05 was considered significant.

Acknowledgements

This work was supported by NIH Grant AI046964, the Rockefeller Brothers Fund and the Edward J. Malinckrodt, Jr. Foundation (VN).

References


**Fig. 1. Genetic analysis of the GAS sag operon for SLS biosynthesis:**
I designed and generated all the mutants in the study.

**Fig. 2. Site-directed mutagenesis studies of the SagA gene product**
I have designed and made all the site directed mutations in the pDCSagA vector to study essential amino acid residues for active SLS expression.

**Fig. 3. Analysis of other virulence phenotypes in wild-type and sagA mutant GAS**
I do greatly appreciate the assistance Dr. R. Kansal and Dr. M. Kotb for their experimental contribution in immunoblot analyses for SpeB and M protein and the assay for SpeB activity (Fig. 3A&B). I determined the Hyaluronic acid capsule (Fig. 3C).

**Fig. 4. Contribution of SLS to M1T1 GAS necrotizing soft tissue infection**
I took a lead role in design and execution of all in vivo GAS infections studies, including assessment of the infection, quantitative culture, S. M. Myskowski provided technical assistance in these studies.

**Fig. 5. Histopathology of skin and subcutaneous tissues at 96 h post challenge**
Skin biopsies were collected in the above mentioned experiment. All tissue biopsies were processed under direct supervision of Dr. N. Varki. I thank Dr. Varki for all her efforts in reading histopathology of the GAS induced necrotic lesion in murine model.

**Fig. 6. Anti-phagocytic properties of streptolysin S**
I have executed the experiments of whole blood survival assay using heparinized blood from 8 different donors (Fig. 6A). Neutrophils were purified by me in the neutrophil killing assay and exposed to wild type M1T1 and SLS- mutant (Fig. 6B)

**Fig. 7. Cytolytic activity of streptolysin S (SLS) against human cells**
Human keratinocyte cell lines infection with M1T1 and isogenic SLS-mutant. Cell viability assays using trypan blue was carried out by me(Fig. 7A). I also made the M49 KO and SLO KO and double KO mutants and studied the contribution of SLS in cell injury quantified by LDH assay (Fig. 7B).

I thank L.A. Kwinn and D.N. Chiem for their studies showing SLS does not contribute to epithelial cell invasion or cathelicidin resistance.

Chapter II

The β-hemolytic phenotype of GGS and S. porcinus is produced by exotoxin SLS

A. Introduction

In patients with underlying medical conditions, β-hemolytic group G streptococci can cause severe invasive disease. These infections resemble those produced by group A streptococci. The β-hemolytic phenotype of group G streptococcus is produced by the exotoxin SLS, encoded by a functional homologue of the nine-gene group A streptococcus sag operon. SLS in GGS contributes to the pathogenesis of necrotizing soft tissue infection. This chapter describes the clinical case reports of the necrotizing fasciitis caused by group G streptococci (GGS), and the discovery of GAS homologous sag-locus responsible for the production of SLS in GGS.

The experiment included are

(a) The sequence analysis of first three genes of sag-operon (sagA, sagB and partial N-terminal region of sagC) show an unique homology to sag-locus in GAS.

(b) Plasmid integration mutagenesis in sagA demonstrate that the operon is necessary for SLS expression in GGS.
(c) In vivo challenges in the murine model to prove that SLS in GGS similar to GAS contributes to necrotic skin and soft tissue injury.
Mechanisms of disease

Streptolysin S and necrotising infections produced by group G streptococcus

Deepali Humar, Vivekananda Datta, Darrin J Bast, Bernard Beall, Joyce C S De Azavedo, Victor Nizet

Summary

Background We encountered three patients with severe necrotising soft tissue infections due to β-haemolytic group G streptococcus. Due to strong clinical similarities with invasive infections produced by group A streptococcus, we investigated a potential link of shared β-haemolytic phenotype to disease pathogenesis.

Methods Hybridisation, DNA sequencing, targeted mutagenesis, and complementation studies were used to establish the genetic basis for group G streptococcus β-haemolytic activity. The requirement of group G streptococcus β-haemolsyn in producing necrotising infection was examined in mice.

Findings Each patient had an underlying medical condition. β-haemolytic group G streptococcus was the sole microbial isolate from debrided necrotic tissue. The group G streptococcus chromosome contained a homologue of the nine-gene group A streptococcus sag operon encoding the β-haemolysin streptolysin S (SLS). Targeted mutagenesis of the putative SLS structural gene sagA in group G streptococcus eliminated β-haemolytic activity. Mice injected subcutaneously with wild-type group A streptococcus or group G streptococcus developed an inflammatory lesion with high bacterial counts, marked neutrophil infiltration, and histopathological evidence of diffuse tissue necrosis. These changes were not found in mice injected with the isogenic group A streptococcus or group G streptococcus SL5-negative mutants.

Interpretation In patients with underlying medical conditions, β-haemolytic group G streptococcus can produce necrotising soft tissue infections resembling those produced by group A streptococcus. The β-haemolytic phenotype of group G streptococcus is produced by the exotoxin SLS, encoded by a functional homologue of the nine-gene group A streptococcus sag operon. SLS expression contributes to the pathogenesis of streptococcal necrotising soft tissue infection.

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Introduction

Group G streptococci are commonly part of the normal flora of human skin, pharynx, and gastrointestinal tract.1 Human group G streptococci isolates are subdivided on the basis of colony size and haemolytic phenotype on sheep blood agar. Small colony group G streptococci exhibit variable haemolytic reactions and are classified within the Streptococcus milleri group. Large colony group G streptococcus isolates, now classified as S. dysgalactiae subspecies S. equisimilis,2 produce robust β-haemolysis and are morphologically very similar to the prominent pathogen group A streptococcus.

Since the mid-1980s, an increase in life-threatening invasive infections produced by group A streptococcus has been well documented.3-4 Prominent among these syndromes is necrotising fasciitis, a destructive...
infection of the subdermal soft tissues frequently complicated by toxic shock syndrome.² By contrast, serious group G streptococcus infections occur only rarely, including endocarditis,³ septic arthritis,⁴ bacteremia,⁵ and septic shock.⁶ We identified only one published case each of necrotising fasciitis or myositis caused by group G streptococcus.⁷ ⁸ ⁹

Here we report three cases of severe necrotising infections due to β-haemolytic group G streptococcus (panels 1, 2, and 3). Because of similar clinical presentations to group A streptococcus infections, we investigated a link between bacterial β-haemolysin phenotype and disease pathogenesis. We used molecular techniques and a murine infection model to identify the β-haemolysin of pathogenic human group G streptococcus, and assess its contribution to disease pathogenesis.¹⁰

Methods

Group G streptococcus isolates were identified by the API 20 Strept identification system (bioMérieux, St Louis, MO, USA). Published methods were used for M-protein (cmm) genotyping,¹¹ T-antigen typing,¹² opacity factor testing,¹³ and pulsed-field gel electrophoresis (PFGE) analysis.¹⁴ Haemolytic titres were determined in a liquid-phase assay¹⁵ in aerobic growth conditions. We used culture and transformation conditions as previously described.¹⁶ The group G streptococcus isolate from Case 1 (VASD1) was selected for genetic and animal virulence studies. We did dot-blot hybridisation analysis with digoxigenin-labelled group A streptococcus 5′ gene probes from the nine-gene operon encoding the β-haemolysin streptolysin 5 (SLS). A 2.4 kb HindIII fragment of group G streptococcus chromosomal DNA probe that was positive for sgaA by

Panel 3

Case 3: A 58-year-old headache man with ethanol-induced cirrhosis and chronic lower extremity lymphoedema was admitted with a 3 day history of left thigh pain. Temperature was 30°C and blood pressure 70/50 mm Hg. His thigh was tender and erythematous from knee to groin with bullae formation. He was treated with cefazolin and clindamycin. He developed progressive jaundice, hypotension, and coagulopathy. Intravenous immunoglobulin was administered. On day 3 he developed swelling and erythema of the left knee and left wrist, and numerous focal necrotic skin lesions of his digits. Gross examination of the left thigh fascia showed extensive liquefaction necrosis. Biopsy samples showed necrotising fasciitis with contiguous myonecrosis. Septic arthritis of the left knee and left wrist were present, with fascial necrosis extending into the left extensor forearm. Extensive drainage and debridement were done. Blood, knee, wrist, thigh fascia, forearm fascia, and skin lesion cultures all grew group G streptococcus. Echocardiogram was normal. He developed adult respiratory distress syndrome, pneumonia, and candidemia. He died on day 12 despite aggressive supportive care.

Figure 1: Histopathology of debrided tissue from patient with group G streptococcus necrotising fasciitis of the calf

Extensive tissue necrosis, vascular thrombosis (arrow), and neutrophilic infiltration can be seen on (A) low power (40x) and (B) high power (100x) views of the haematoxylin and eosin stained sections.
Southern blot analysis was cloned in Escherichia coli and sequenced directly.

For mutagenesis studies, we amplified an intragenic fragment from the group G streptococcus sagA gene using PCR and cloned in temperature-sensitive vector pHY304. This knockout plasmid was introduced into group G streptococcus by electroporation, and transformants selected at 30°C. Homologous recombination events in the group G streptococcus chromosome were identified by shifting to the non-permissive temperature (37°C) while maintaining antibiotic selection. Fidelity of site-directed recombination event was confirmed by PCR. Complementation was done as follows: the group G streptococcus sagA knockout mutant was rendered competent and transformed with vector pSagLocus containing the SLS operon genes of group A streptococcus.

We tested group G streptococcus virulence in a mouse model of streptococcal necrotising fasciitis. Briefly, 10^6 cfu of log-phase bacteria were mixed with Cytodex beads (Sigma) and injected subcutaneously into the right flank of hairless 4-week-old male c57Bl/6j/hr/hr/B mice (Charles River, Wilmington, MA, USA). Six animals were tested for the group G streptococcus parent strain and its isogenic sagA mutant; group A streptococcus strain NZ131 (M49) and its corresponding sagA mutant were tested for comparison. Animals were monitored for development of necrotic ulcers, and killed at 24 h or 48 h for quantitative culture and histopathological assessment. Data were compared using the exact Wilcoxon rank-sum test.

Role of funding sources
The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing the report.

Results
Each group G streptococcus isolate from the patients was S. dysgalactiae, subspecies Equinsilis, with T-antigen type 25 and negative opacity factor. Group G streptococcus isolates from Cases 1 and 2 possessed M-protein (cmn) gene stg480 (GenBank Accession number X79520) previously identified in group G streptococcus isolates from bloodstream and wound infections. Group G streptococcus isolates from Case 3 possessed the cmn gene stc74a (GenBank Accession number X60097) which has been found in various β-haemolytic human group C streptococcus and group G streptococcus isolates. PFGE analysis showed that the group G streptococcus from Cases 1 and 2 were clonal, whereas the isolate from Case 3 was genetically distinct. No direct or common-source epidemiological contacts among the three patients were identified. The in-vitro haemolytic activities of the three invasive group G streptococcus isolates (mean titre 149 HU) were similar to those of group G streptococcus strains isolated locally from epithelial sites (147 HU), and greater than a panel of 13 group A streptococcal strains of differing M-protein serotype provided by the Centers for Disease Control (23 HU).

We examined chromosomal DNA from strain VASD1 (from patient 1) and four other β-haemolytic group G streptococcus strains for homologies to the nine genes (sagA–sagI) in the group A streptococcus operon encoding SLS activity. By means of dot-blots analysis, strong hybridisation signals were obtained from group G streptococcus DNA with probes for each of the nine group A streptococcal genes (data not shown). DNA sequencing showed that the sag operon promoter and a terminator motif responsible for differential transcription of sagA versus sagβ-I were highly conserved. Comparison of predicted protein sequences for the group G streptococcus and group A streptococcus gene products showed 89% identity and 94% similarity for SAGA (figure 2), 81% identity and 92% similarity for Sagβ, and 74% identity and 81% similarity for the N-terminus of SagC, respectively. Confirmed plasmid integration mutagenesis of sagA yielded a group G streptococcus with no detectable β-haemolytic activity (figure 3). β-Haemolytic activity could be partially restored to the group G streptococcus sagA mutant by introduction of the intact group A streptococcus sag loci on a plasmid vector (figure 3). These studies show that a functional homologue of the group A streptococcus SLS (sag) operon is present in group G streptococcus. The new group G streptococcus sequence information has been submitted to the DDBJ/EMBL/GenBank databases under the accession number AJ039399. We did additional sequencing of the sagA gene on a group G streptococcus bloodstream isolate (T107) from the Toronto Invasive Bacterial Diseases Network (TIBD) collection. This strain had a gene 100% homologous to sagA from group G streptococcus patient isolate VASD1. Like group G streptococcus, large-colony group G streptococcus exhibiting robust β-haemolysis are classified within S. dysgalactiae subspecies equinsilis.

Figure 2: Comparison of the deduced amino-acid sequences of SagA between group G streptococcus (GGS) and group A streptococcus (GAS)
**Table 1.** Necrotic ulcer formation and wound culture log values for bacterial strains tested in mice.

<table>
<thead>
<tr>
<th>Bacterial strain tested</th>
<th>Necrotic ulcer formation (24 h)</th>
<th>Wound culture (cfu/gm) mean log value (24-48 h)</th>
<th>Wound culture (cfu/gm) range (24-48 h)</th>
<th>Neutrophilic infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group G wildtype</td>
<td>100%</td>
<td>6.45</td>
<td>1.9×10^9-4.1×10^9</td>
<td>++++</td>
</tr>
<tr>
<td>Group A wildtype</td>
<td>100%</td>
<td>7.34</td>
<td>1.3×10^9-6.7×10^9</td>
<td>++++</td>
</tr>
<tr>
<td>Group G sgaA mutant</td>
<td>0</td>
<td>3.97†</td>
<td>1.0×10^9-5.9×10^9</td>
<td>+</td>
</tr>
<tr>
<td>Group A sgaA mutant</td>
<td>0</td>
<td>4.93‡</td>
<td>7.7×10^8-1.2×10^9</td>
<td>+</td>
</tr>
<tr>
<td>Cylodex alone</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
</tr>
</tbody>
</table>

Six mice were tested in each group. *P<0.0022 vs wildtype. †P<0.005 vs wildtype. Exact Wilcoxon rank-sum test.

### Effect of streptolysin S gene mutation on group G streptococcus and group A streptococcus virulence in the murine model of necrotising fasciitis

The contribution of group G streptococcus SLS expression in the pathogenesis of necrotising fasciitis was tested in mice (table). Within 24–48 h, animals injected subcutaneously with the group G streptococcal clinical isolate developed necrotic ulcers at the site of inoculation, had high bacterial counts on lesion culture, and showed histopathological evidence of diffuse skin and soft tissue necrosis with substantial neutrophil infiltration. By contrast, mice injected with the group G streptococcus sgaA mutant did not develop necrotic ulcers, had ten-fold lower bacterial counts on lesion culture (P=0.0022), and showed minimal degrees of tissue injury or neutrophil infiltration. Representative gross and microscopic pathological findings are shown in figure 4. The results were similar to those seen with the group A streptococcus M49 strain and sgaA mutant used as a control and to our previously reported observations in M1 strains and SLS-negative transposon mutants. Two mice infected with the group G streptococcus sgaA mutant appeared to have cleared the infection by 48 h (<10 cfu/gm tissue). In the four mice without necrotic ulcers but with persistence of bacteria at the inoculation site, up to 20% of the recovered colonies had reverted to the wild-type β-haemolytic phenotype. The latter finding suggests an in vivo selective pressure toward excision of the integrative plasmid through reverse homologous recombination.

### Discussion

We report three patients with necrotising soft tissue infections resembling group A streptococcus disease in which the sole microbial isolate was β-haemolytic group G streptococcus. A severe underlying medical condition was present in each case. Diabetes mellitus, malignancy, and cirrhosis are commonly reported risk factors for development of other types of invasive group G streptococcus infection. Despite initial therapy with intravenous antibiotics active against group G streptococcus, all three patients had clinical deterioration and grew viable organisms from the necrotic tissues when surgery was done. This observation reinforces the importance of prompt and thorough surgical debridement for the successful therapy of streptococcal necrotic fasciitis.

The β-haemolysin of human pathogenic group G streptococcus and group C streptococcus is SLS, encoded by a nine-gene operon highly similar to that recently discovered in group A streptococcus. The

![Figure 4](image-url) **Figure 4:** Representative gross and microscopic histopathological findings in mice infected subcutaneously with a group G streptococcal necrotising fasciitis clinical isolate (A) versus an isogenic streptolysin S-deficient mutant (B).

Ulcus formation with necrotic tissue destruction, vascular thrombosis, and diffuse neutrophilic infiltrate are noted with the wild-type strain, whereas only minimal inflammatory changes are seen with the mutant.
group G streptococcus SLS precursor, SagA, retains key features of this bacteriocin-type toxin, including a predicted Gly-Gly cleavage site to yield a propeptide matching the calculated size (2.8 kD) of mature SLS.\(^1\)\(^2\)\(^3\) Downstream genes, including the parative modifying enzyme sagB and ATP-binding cassette exporter sagG-I are also conserved.\(^4\)\(^5\) Targeted mutagenesis of the group G streptococcus sagA gene abolishes β-haemolytic activity, and this phenotype is partially restored upon transformation of the mutant with the group A streptococcus homologue. These data confirm that genes of the sag operon are both necessary and sufficient for SLS production.

The bacteriocin-like SLS precursor SagA shares no homology whatsoever with streptolysin O (SLO), a 57-kD thiol-activated cytolytic expressed by group A, C, and G streptococci, for which the gene has been identified.\(^6\)\(^7\) By contrast with SLS, SLO is inactivated by oxygen, inhibited by cholesterol, produces little to no detectable haemolysis on blood agar plates, is immunogenic, and oligomerises in the red-blood-cell membrane to form a pore. The β-haemolysin and virulence factor of the important human pathogen group B streptococcus is unrelated to SLS or SLO, but rather seems to be encoded by a new 78.3 kD gene, CylE.\(^8\)\(^9\)\(^10\) An interesting comparison for SLS may be the plasmid-encoded haemolysin/cytolysin expressed by 45–60% of Enterococcus faecalis (formerly Group D streptococcus) isolates from patients. Although sharing little primary sequence similarity to SagA, this small protein toxin is composed of two structural subunits that belong to the antibiotic class of bacteriocin peptides.\(^11\)\(^12\)\(^13\)

In contrast to the wild-type parent strains, SLS-negative sagA mutants of group G streptococcus and group A streptococcus are not virulent in a murine model of streptococcal necrotising fasciitis. These findings suggest that SLS expression is required for the pathogenesis of this destructive infection. SLS is one of the most potent cytotoxins known,\(^14\)\(^15\) capable of injuring a wide array of membranes including those of lymphocytes, neutrophils, and certain tissue culture cell lines.\(^16\)\(^17\)\(^18\) The precise mechanisms of SLS membrane toxicity are not known. SLS does not seem to possess phospholipase action, and electron microscopic examinations of erythrocyte membranes damaged by SLS do not show large pores such as those induced by SLO.\(^19\)\(^20\) We hypothesise that SLS contributes to the development of streptococcal necrotising fasciitis via direct toxicity to cells of the deep soft tissues and feeding vessels, leading to cell death and provoking neutrophil influx. In vitro and primate model studies with group A streptococcus suggest that neutrophil-derived reactive oxygen metabolites and proteases may act together with bacterial cytotoxins to accelerate necrotic fascial injury.\(^21\)\(^22\) SLS-mediated neutrophil lysis could cause release of such factors and prevent phagocytosis, explaining in part how wild-type group A streptococcus and group G streptococcus persist in the infection model despite intense neutrophil recruitment.

SLS toxin is clearly not sufficient to trigger necrotising fasciitis. SLS is produced by virtually all group G streptococcus and group A streptococcus isolates, even those isolated from asymptomatic individuals. Moreover, we found SLS haemolytic activity levels to be greater in group G streptococcus than the more virulent group A streptococcus. Human β-haemolytic group G streptococcus share many other important virulence factors with group A streptococcus including the antiphagocytic surface M and M-like proteins, streptokinase, fibronectin, and IgG binding proteins, SLO, C5a peptidase, NADase, and possibly a hyaluronic acid capsule.\(^23\)\(^24\) Of notable exception are the absence in group G streptococcus of the group A streptococcus pyrogenic exotoxins SPE-A—the scarlet fever toxin A, and SPE-B, a chromosomally-encoded cytolytic proteinase. SPE-A production is strongly linked epidemiologically with strains identified in the present resurgence of invasive group A streptococcus infections.\(^25\) As we show with SLS, production of SPE-B, M-protein, and hyaluronic acid capsule are known to contribute to development of group A streptococcus necrotising fasciitis in the murine model.\(^26\)\(^27\) Absence of pyrogenic exotoxins or differences in the coordinate regulation of virulence factor expression\(^28\)\(^29\) may account for the apparent inability of group G streptococcus to produce necrotising fasciitis in the non-compromised host.

Strategies aimed at neutralisation of SLS activity could be of therapeutic benefit as adjuncts to definitive surgical and antibiotic management of streptococcal necrotising fasciitis.

Contributors
D. Humar and V. Nizet reported the clinical cases. V. Dutta, D. Humar, DJ. Basta, JCS De Araujo, and V. Nizet designed and carried out the molecular genetic and in vivo experiments. B. Beal performed the enzymes-gotyping and PFGE analysis. D. Humar, V. Dutta, and V. Nizet prepared the original manuscript. All authors contributed to the revised manuscript. D. Humar and V. Dutta contributed equally to this work.

Conflict of interest statement
None declared.

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References


Chapter II B, in full, is a reprinted of the material as it appears in Datta V*, Humar D*,
C. Production of Streptolysin S by *Streptococcus porcinus*

C1. Abstract

*Streptococcus porcinus* is a β-hemolytic streptococcal species that has been associated with serious infections in swine. Reports of *S. porcinus* isolation from human sources are on the rise, ranging from asymptomatic rectovaginal carriage to rare cases of neonatal bacteremia or puerperal sepsis. Here we describe the discovery of the genetic basis for β-hemolysis in *S. porcinus*, revealing the phenotype is produced by a homologue of streptolysin S.

C2. Introduction

*Streptococcus porcinus* has been long associated with debilitating diseases in swine ranging from septicemia, endocarditis to lymphadenitis (Katsumi 1998). The organism is recognized to produce clear zones of β-hemolysis when cultured on blood agar media (Shuman 1974; Loubinoux, Mihaile-Amrouche et al. 2004).

Rarely has *S. porcinus* been associated with human disease; however a series of newborn bacteremias in the 1980s were proven to be caused by isolates *S. porcinus* (Facklam 1995). Further investigation revealed that women of child bearing age can be asymptomatic carriers of bacterium (Facklam 1995). An additional recent report attributed a case of spontaneous abortion in a 22-week gestation, previously uncomplicated pregnancy, to placental invasion by *S. porcinus* (Martin 2004). These cases are reminiscent of the pathogenesis and clinical spectrum of the β-hemolytic
group B *Streptococcus* (GBS, *S. agalactiae*), the leading cause of invasive bacterial infections in human neonates and the target of universal screening and intrapartum antibiotic prophylaxis (Ferrieri 2000).

*S. porcinus* has also been isolated from women with perpeurl sepsis, or “childbirth fever” (Facklam 1995). Similar infections can be produced by GBS (Matorras, Garcia-Perea et al. 1991), but were historically associated with another major human pathogen, the group A *Streptococcus* (GAS, *S. pyogenes*) (Nathan, Peters et al. 1993).

The genetic bases of β-hemolysin production in GAS and GBS have recently been discovered, and interestingly, the toxins responsible for the phenotype in each species are wholly unrelated (Nizet 2002). The small bacterioein-like peptide responsible for β-hemolysis in GAS is streptolysin S (SLS), encoded by a 9-gene operon termed *sag* for “streptolysin-associated genes” (Nizet, Beall et al. 2000; Datta 2005). Homologues of the *sag* operon are present in other species including groups G and C *Streptococcus* (GGS & GCS) (Humar, Datta et al. 2002) and fish pathogen *S. iniae* (Fuller, Camus et al. 2002).

In contrast, the single gene necessary and sufficient for expression of the β-hemolysin of GBS is cylE (Spellerberg, Martin et al. 2000; Pritzlaff, Chang et al. 2001), encoding a predicted 79 kD protein without GenBank homologies. The respective β-hemolysin toxins are important virulence factors in the pathogenesis of GAS and GBS infections.
In this paper, we use low stringency polymerase chain reaction, DNA sequencing, targeted mutagenesis, and complementation analysis to identify the β-hemolysin of *S. porcinus* as a novel member of the SLS toxin family.

**C3. Materials and Methods**

Standard microbiological and chemical methods were used for streptococcal species identification (Facklam 1995; Koneman E.W. 1997; Thompson and Facklam 1997), including further confirmation by DNA sequence for the 16s ribosomal subunit as described (Facklam 1995). The single primer PCR technique for amplification of *S. porcinus* genomic DNA was initially performed using primers from the known *sag* operon sequence of GAS (Nizet, Beall et al. 2000), then extended with new primers from the derived *S. porcinus* sequence. The amplified products were T-A cloned in vector pCR2.1-TOPO (Invitrogen) and sequenced in both directions using primers in the cloning vectors. The sequence contigs were assembled using the BioEdit program. Mutagenesis studies were carried out by PCR amplification of an intragenic fragment of the *S. porcinus sagA* gene which was captured in pCR2.1-topo then subeloned into the multiple cloning site of temperature-sensitive *E. coli*-streptococcal shuttle vector pHY304 (Yim, Nittayarin et al. 1997), to produce targeting vector pSagA-KO. Wild-type *S. porcinus* strain C2244 was transformed with the pSagA-KO and transformants identified under erythromycin selection at the permissive temperature of 30°C. Campbell-type single crossover, chromosomal integrational events were selected by maintaining the bacteria under erythromycin selection and shifting the culture to 37°C,
the nonpermissive temperature for plasmid replication. The fidelity of the site-directed plasmid integration was confirmed by PCR. Complementation studies were performed by introducing the vector pSagLocus (Nizet, Beall et al. 2000; Humar, Datta et al. 2002) expressing the GAS SLS biosynthetic operon in the \textit{S. porcinus} \Delta\textit{sagA} mutant.

\textbf{C4. Results}

Six unusual β-hemolytic streptococcal isolates were collected from five patients enrolled in a multicenter studies of GBS colonization. The isolates from two patients were present in cocolonization along with common isolates of GBS in recto-vaginal swabs (Ferrieri 2000). Each of the isolates was found to be non-reactive to anti-GBS sera by Ouchterlony double-diffusion assay. Microbiologic assays on the isolates found them to be CAMP factor positive, with weak hippurate hydrolysis, positive bile-esculin hydrolysis, weakly-positive on PYRase test, and bacitracin negative. The above mentioned test results along with positive arginine hydrolase test, Voges-Proskauer positive and mannitol fermentation, identified the clinical isolate as \textit{S. porcinus}, as per the microbiological identification of the strains from pigs (Katsumi 1998). As these isolates were cultured from patients enrolled in GBS colonization studies, a 16S rRNA sequence studies were carried out to confirm the identification of \textit{S. porcinus} (Thompson and Facklam 1997). 16S rRNA sequence matched 98% with the \textit{S. porcinus} from bases 174-1447 confirming their assignment within this species (Ferrieri 2000). As reported in previous studies (Thompson and Facklam 1997), the observed zone of
β-hemolysis produced by these *S. porcinus* strains were greater than typically seen with GAS or GBS human isolates.

Using PCR under low stringency conditions (45°C annealing temperature), a small fragment of DNA was amplified from the *S. porcinus* chromosome with forward and reverse primers from the GAS *sagA* sequence. Similar low stringency PCR using two different primer sets from the GBS *cylE* sequence failed to amplify products from the *S. iniae* chromosome. The amplified *S. porcinus* sequence was used to design new primers for single primer PCR walking upstream and downstream to provide the complete sequence of the *sag* promoter, the full *sagA* ORF, the rho-independent terminator sequence, and the beginning of a *sagB* ORF.

Comparative analysis of the predicted SagA peptide sequence from *S. porcinus* with the known SagA peptide sequence of GAS showed 88% identity and 94% similarity, including the characteristic PGG cleavage site for a bacteriocin-like toxin followed by a string of six cysteine residues to begin the propeptide domain compared to five in GAS (Fig. 5). Examining the alignment at of the known predicted SLS precursor molecules, including those of GGS and GCS (Humar, Datta et al. 2002) and *Strept. iniae* (Fuller, Camus et al. 2002), several additional common features are clear including overall size, enrichment in threonine, serine and glycine residues, and an invariant terminal lysine (Fig. 5). Curiously, the leader sequence appears to be very highly conserved, while more variation across species is seen in the propeptide destined to become processed into the mature β-hemolysin SLS toxin.
To determine if the sagA gene homologue identified in *S. porcinus* was indeed solely responsible for the organism’s β-hemolytic phenotype, targeted plasmid integrational disruption of the sagA ORF was performed. This mutagenesis eliminated the β-hemolytic activity of the organism, and the phenotype could be restored by heterologous expression of the cloned sag locus from GAS (Fig. 6). These results indicate that β-hemolysis in *S. porcinus* is the byproduct of a SLS homologue.

**C5. Discussion**

Very little clinical data is available regarding human *S. porcinus* infections; rather this pathogen is primarily associated with disease in swine. The majority of human strains reported in the largest published series (9 of 13) were isolated from the genitourinary tract of females of childbearing age, including cases of perpueral sepsis, newborn bacteremia, and spontaneous abortion (Facklam 1995; Martin 2004). β-hemolytic *S. porcinus* have been characterized by their cultural and biochemical properties, determination of antibiotic susceptibility and serological classification (Lammler 1998). In this study, we analyzed a human isolate genitourinary isolate of *Strep. porcinus* with a broad zone of β-hemolysis and positive for CAMP factor. In their natural environment in swine, *S. porcinus* may cohabitate along side of other β-hemolytic streptococci including GCS and GGS which possess known homologues of the sag operon for SLS production (Katsumi 1998; Humar, Datta et al. 2002). In their human environment, they may frequently encounter the pathogen GBS which
possesses a different mechanism for β-hemolysin production, with the cy/E gene representing the key genetic determinant (Nizet 2002).

Our targeted mutagenesis studies reveal the β-hemolysin of S. porcinus to be SLS, encoded by the nine gene operon originally discovered in GAS (Nizet, Beall et al. 2000), and later found in other β-hemolytic streptococci including GCS, GGS and S. iniae (Fuller, Camus et al. 2002; Humar, Datta et al. 2002). The predicted S. porcinus SLS structural gene product SagA retains the key features of these bacteriocin-like toxins, including the characteristic PGG cleavage site to release a predicted 30 amino acid propeptide. This mature peptide matches with the predicted 2.8KDa size of SLS in a partially purified extract of the GAS hemolysin (Nizet, Beall et al. 2000). Although the leader domains of the two proteins were extremely conserved, the cleaved propeptide possessed a few differences. Also the sequence of the downstream DNA found a rho-independent terminator more similar to GGS terminator (15 bp repeats vs 17 bp in GAS)(Nizet, Beall et al. 2000). The comparative higher hemolytic activity of streptolysin S in GGS has already been reported (Humar, Datta et al. 2002), and perhaps greater read through in the smaller hairpin terminator may in part be responsible for the same phenomenon in S. porcinus. We have not yet tested the role SLS plays in the pathogenesis of S. porcinus, but we hypothesize that it will have similar effect in a model of invasive soft tissue infection as seen in GAS, GGS and S. iniae (Fuller, Camus et al. 2002; Humar, Datta et al. 2002; Datta 2005). It is important to note that SLS may not be sufficient in itself for the development of invasive infection, as the synergistic effects of other known GAS virulence factors like M-
protein, streptokinase, cysteine protease, pyrogenic exotoxins and others described in GAS may account for the highest virulence potential of this pathogen. Nevertheless, it is apparent that _S. porcinus_ may represent an emerging infectious disease agent in certain susceptible human disease populations such as pregnant women and newborn infants.
Fig. II. 5: The β-hemolysin of *S. porcinus* is a streptolysin S homologue. SLS in *S. porcinus* is encoded by the *sagA* gene as confirmed by targeted mutagenesis and complementation analysis.
Fig. II. 6: Comparitive study of the SagA protein from different β-hemolytic Streptococci. Deduced protein sequences of the predicted SagA gene products representing the predicted prepropeptide precursors of streptolysin S from GAS, GCS/GGS, Streptococcus porcinus, and Streptococcus iniae.
Fig. 1. Histopathology of debrided tissues from the patient with group G streptococcal necrotizing fasciitis of the calf.
Dr D. Humar(DH) reported the clinical cases of necrotizing fasciitis caused by group G Streptococcus. I did not participate in this studies.

Fig. 2. Comparison of the deduced amino-acid sequence of SagA between group G Streptococcus(GGS) and group A Streptococcus(GAS).
Sequencing and submission of the sequence in the genome bank was carried out by me, I also compared the sequence homologies of SagA in GGS and GAS.

Fig. 3. Elimination of group G streptococcus β-hemolysin by mutation of sagA and partial complementation.
Under my direct supervision DH and I have used molecular genetics technique to create mutation in sagA of GGS. Partial complementation was carried out by me.

Fig. 4. Representative gross and microscopic histopathological findings in mice infected subcutaneously with GGS and its isogenic SLS negative mutant.
I took a lead role in design of all the in vivo GGS infection studies; I thank Drs D.J. Bast and J.C.S. de Azavedo for their assistance and expertise in our murine challenges.

I thank Dr. B. Beall for providing us several GGS isolates for comparative hemolytic assay.

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REFERENCES


Chapter III

Induction of proinflammatory cytokine by SLS and inhibition of SLS using phospholipid dipalmotyl phosphatidylcholine (DPPC)

A. Introduction

Rise in incidence of the severe invasive GAS disease and its association severe toxicity and multiorgan failure. The toxicity is due to the insurge of proinflammatory cytokine has been linked to superantigenic properties of Streptococcal pyogenic exotoxins circulating in the host. Streptolysin S (SLS) has been demonstrated to play an important role in the pathogenesis of M1T1 GAS necrotic infection in mice (Chapter II). Skin biopsies from mice infected with M1T1 GAS show necrotic ulceration with neutrophilic infiltration to the site of infection, while these histopathological findings were not seen in mice challenged with SLS-negative isogenic mutants. These results prompted us to investigate the role of SLS in the induction of proinflammatory cytokine like TNFα and also the upregulation of nitric oxide synthetase (iNOS) gene.

The destructive injury to the soft tissues in NF patients and poor out come of the patient including amputation of limbs or death, despite an aggressive therapeutic approach. As demonstrated in Chapter II of this dissertation, contribution of SLS in GAS virulence includes cytotoxicity to human epithelial cells, resisting phagocytic clearance, and challenges of wild-type GAS in murine model causes necrotic lesion and bacteremia. Cytolytic effects of impure SLS extracts have been inhibited using trypan blue or certain phospholipids using in vitro model system, but inhibition of cytotoxic
effect of SLS by a natural phospholipids dipalmotyl phosphatidylcholine (DPPC) has not been studied. In this section of the chapter we designed experiments to study the inhibitory effects of DPPC. The experiments designed to study protection against SLS are

(a) Protective effects of phospholipids in epithelial tissue culture cells exposed to wild type M1T1 GAS bacteria.

(b) Inhibition of antiphagocytic property of SLS by DPPC

(c) DPPC as therapeutic agent in the control of GAS necrotic skin infection in murine model
B. GAS SLS induction of macrophage TNFα and iNOS production

B1. Introduction

Recent decades have seen an increasing incidence of the severe invasive GAS disease called necrotizing fasciitis (NF) (Davies et al., 1996; O'Brien et al., 2002). GAS NF is a rapidly progressing bacterial infection in the facial planes usually associated with bacteraemia, severe toxicity and multiorgan failure, often leading to a poor clinical outcome (Bouvet, 2001; Efstratiou, 2000). Histopathologic examination of biopsies obtained during surgical intervention of GAS NF reveal extensive necrosis of the subcutis which usually extends to all the dermal layers of the skin (Brogan and Nizet, 1997; Cunningham et al., 2001; Schwartz et al., 1995). Also notable is mixed inflammatory cell recruitment with abundant neutrophils found scattered among the proliferating bacteria in the NF lesion (Bouvet, 2001).

Macrophages and neutrophils are important elements in innate immunity as they recognize, phagocytose and destroy pathogens. It has been demonstrated that the bacterial burden and the severity of the GAS tissue infection can be correlated to proinflammatory cytokine expression at the site of infection (Norrby-Teglund et al., 2001). Cytokines IL-1α, IL-1β, IL-1ra, IL-2, IL-4 IL-8, IL-12, TNF-α, TNF-β, and INF-γ have been detected in the skin biopsies of patients with NF (Norrby-Teglund et al., 2001). GAS superantigens such as SpeA, SpeF and others trigger proinflammatory cytokine production at the site of infection which could enhance the migration of antigen presenting cells to the site (Kotb, 1995; Norrby-Teglund et al., 1994; Norrby-Teglund et al., 2001). Adhesion of GAS to keratinocytes has been
involved in the expression of proinflammatory cytokines like IL-1, which in turn is responsible for expression of IL-6 and IL-8 (Wang et al., 1997). However, it has been postulated that adherence alone is not sufficient to induce proinflammatory cytokine modulation in keratinocytes, but that the action of other GAS secreted products is required (Wang et al., 1997). Inactivation of the pore-forming toxin Streptolysin O (SLO) did reduce the transcription of IL-1β, IL-6 and IL-8, but did not alter the expression of IL-1α (Ruiz et al., 1998).

Another pore-forming toxin of GAS, streptolysin S (SLS), is an oxygen stable β-hemolysin now demonstrated to be critical in the pathogenesis of M1T1 GAS necrotic infection in mice (Datta et al., 2005). Histopathological examination of skin biopsies from mice infected with the wild-type bacteria show a necrotic ulceration with abundant neutrophilic infiltration to the site of infection, while these changes are absent in animals challenged with an isogenic SLS-deficient mutant (Datta et al., 2005). These studies provide suggestive evidence that SLS could also play an important role in induction of proinflammatory cytokines that in turn could be responsible for the recruitment of neutrophils at the site of infection.

B2. Materials and Methods

a. Bacterial strains and media:
GAS strain 5448 is a serotype M1 isolate from a patient suffering from NF and streptococcal toxic shock syndrome (Kansal et al., 2000). The isogenic SLS-deficient mutant M1ΔSagA was produced by in-frame allelic replacement of the SLS structure gene (Timmer et al., 2005). Both strains were cultured in Todd Hewitt Broth (THB, Difeo).

b. Isolation of bone marrow (BM) derived macrophages and stimulation of macrophages:

Macrophages were isolated from BM by collecting the marrow of femur and tibia of C57BL6 mice. Cells were plated on a Petri dish in DMEM cell culture media supplemented with 10% fetal bovine serum (FBS) and 30% conditioned media. Conditioned media was prepared by collecting the supernatant of L-929 fibroblast cells secreting M-CSF. The BM macrophages matured in 7 days becoming adherent to the Petri dish. Adherent macrophages are harvested by gentle scraping and a consistent number (~3x10⁵) of macrophages were plated on to 6 well plates (Corning) for the bacterial infection studies. The cells were exposed to wild-type M1 GAS and the isogenic SLS-deficient mutant at a multiplicity of infection (MOI) of 10 bacteria/macrophage for 1h. The antibiotics penicillin (5mg/ml) and gentamycin (100µg/ml) were then added to the medium to eliminate extracellular bacteria. Cells were harvested at 4h and 24h for total RNA isolation.

c. Reverse transcription and real-time quantitative PCR:
First strand synthesis was achieved from 1 μg of total RNA isolated with Trizol reagent (Molecular Research Center, Inc.) using the SuperScript system (Invitrogen) employing random primers. For real-time PCR (RT-PCR) analyses, cDNAs were diluted to a final concentration of 10 ng/μl and amplified in a TaqMan-Universal Mastermix SYBR Green (Applied Biosytems). Fifty ng of cDNA was used as template to determine the relative amount of mRNAs by RT-PCR in triplicate (ABIPrism 7700 sequence detection system), using specific primers with the following sequences:

\[
\begin{align*}
i\text{NOS forward} & \quad 5'\text{-ACCCTAAGAGTCACAAAATGGC}-3' \\
i\text{NOS reverse} & \quad 5'\text{-TTGATCCTCACATACTGTGGACG -3'} \\
\text{TNF} \alpha \text{ forward} & \quad 5'\text{-CCATTCTGAGTTCTGCAAAGG}-3' \\
\text{TNF} \alpha \text{ reverse} & \quad 5'\text{-AGGTAGGAAGGCGCTGAGATCTTATC}-3'
\end{align*}
\]

B3. Results

The robust inflammatory response produced by wild-type GAS infection is clearly evident in histopathology from the mouse model of NF (Timmer et al., 2005). Since there was diminished neutrophil infiltration in mice infected with the SLS-deficient GAS mutant, we chose to study the induction of proinflammatory cytokine
TNFα by SLS. We infected BM derived macrophages with wild-type and ΔSLS GAS and collected total RNA 24h post-exposure. The wild-type strain was found to induce approximately 4-fold greater production of TNFα message compared to the SLS-deficient mutant (Fig. III. 1).

The generation of nitric oxide facilitates bacterial killing by macrophages and can be linked to increased transcription levels of the inducible nitrous oxide synthetase enzyme (iNOS) (Lirk et al., 2002). We observed a marked induction of iNOS transcription in macrophage monolayer stimulated with wild-type GAS, whereas exposure to the SLS-deficient mutant did not increase the transcriptional levels of iNOS beyond than seen in non-stimulated control macrophages (Fig. III. 2).

**B4. Discussion**

SLS confers a hallmark feature used in identification of the GAS bacterium, namely a marked zone of β-hemolysis around the colony on blood agar media. A potent pore-forming toxin, SLS is associated with the pathogenesis of NF in a murine model of M1T1 GAS infection (Datta et al., 2005). These results are corroborated by findings in skin infection caused by other β-hemolytic streptococci possessing SLS homologues such as GGS and S. iniae (Fuller et al., 2002; Humar et al., 2002). The histopathological findings in the mouse model of NF produced by M1T1 GAS show an large infiltration of neutrophils to the site of infection (Datta et al., 2005). The animals challenged with SLS-deficient mutants did not demonstrate necrotic ulceration and had minimal neutrophil recruitment (Datta et al., 2005; Humar et al., 2002).
TNFα is involved in the augmentation of inflammatory responses to bacterial infection. Moreover, patients receiving anti-TNFα therapy have been reported to be susceptible to GAS-induced NF (Chan et al., 2002). We examined the induction of TNFα in BM-derived macrophages stimulated with wild-type GAS and an isogenic SLS-deficient mutant. SLS pore-forming toxin activity appeared to stimulate macrophages, resulting in an ~7.5 fold induction of TNFα transcription, in contrast to SLS-deficient mutants in which only a 2.5 fold TNFα induction was seen 24h post infection (Fig. III. 1) SLS induction of this potent cytokine might contribute to local amplification of the inflammatory response at the site of infection.

Nitric oxide is known to exert antimicrobial properties against a variety of bacterial species (Lirk et al., 2002). Phagocytosis of Streptococcus pneumoniae by differentiated human monocyte-derived macrophages results in upregulation of inducible nitric oxide synthase (iNOS) (Marriott et al., 2004). Here we observed that exposure of murine BM-derived macrophages to wild-type GAS led to a 800-fold induction of iNOS transcription; in contrast, SLS-deficient mutants have a less than 30-fold increase in iNOS transcription (Fig. III. 2). Induction of iNOS in an SLS-dependent manner may indicate the importance of this pore-forming toxin as a stimulator of the phagocyte. Although the antibacterial effects of NO could promote bacterial clearance, this induction could simultaneously provoke overstimulation of the macrophages which has been correlated to increased apoptosis (Marriott et al., 2004).

Several GAS secreted toxins exhibit superantigenic properties and have been shown to activate T-cells expressing large quantity of cytokines (Kotb, 1995). GAS NF is
usually complicated by the streptococcal toxic shock syndrome (STSS). In patients suffering STSS, circulating GAS superantigens have been detected, which in turn has implicated these factors in the cytokine surge observed in these patients. Our study indicate the pore-forming toxin SLS also equally capable of eliciting proinflammatory cytokines induction in primary macrophages. Upregulation of the Th1-type cytokines like INFγ, TNFα and IL-1 at the site of infection may correspond to the need for proinflammatory factors in recruitment and accumulation of the phagocytic cell infiltrate (Norrby-Teglund et al., 2001).

C. Inhibition of SLS toxicities by phospholipid dipalmotyl phosphatidylcholine (DPPC)

C1. Introduction

The human pathogen GAS causes disease ranging from mild pharyngitis and superficial impetigo (Johnson et al., 1992) to serious infections including NF and STSS (Bisno and Stevens, 1996; Stevens et al., 1989; Stevens, 1992). There appears to be an increasing prevalence of severe, invasive GAS infections approaching epidemic proportions in several regions of the world (Davies et al., 1996; Stevens, 1996). This
destructive injury to the soft tissues, despite an aggressive therapeutic approach, may be associated with poor outcomes including amputation or death (Stevens, 2000). β-lactam antibiotics such as penicillin and cephalosporins are the mainstay for treatment of GAS infection, and pharyngitis and superficial skin infections respond well to oral antibiotic therapy. However, there are growing numbers of clinical treatment failures when severe invasive GAS disease is treated with penicillin, even using high dose intravenous therapy. This failure of penicillin is largely attributed to the physiological state of the organism, wherein penicillin-binding-proteins (PBPs) are not expressed by bacteria in stationary-phase growth (Kotb et al., 1993). The protein synthesis inhibitor clindamycin have been shown to have greater efficacy in treatment of GAS invasive infection when used along with penicillin, and has the theoretical benefit of decreasing bacterial toxin elaboration that may exacerbate disease progression.

The distinct zone of β-hemolysis surrounding GAS grown on blood agar is the result of the potent toxin SLS. It has been observed that the SLS toxin can exist in secreted, intracellular or cell-bound forms (Ginsburg, 1999). As we have shown, the toxin is most likely a bacteriocin-like peptide (Nizet et al., 2000), for which the 9 gene GAS sag locus is necessary and sufficient for production (Nizet et al., 2000). SLS contributes to GAS virulence phenotypes including cytotoxicity to human epithelial cells, impairment of phagocytic clearance, and the production of NF and bacteremia in the murine model (Chapter II publication (Datta, 2005)).

Although humans do not generate antibodies against SLS during the natural course of infection, polyclonal antisera raised against synthetic peptides corresponding
to the C-terminus of the proposed SagA SLS precursor are able to neutralize the
hemolytic action of the toxin (Carr et al., 2001; Dale et al., 2002). Similarly, cytolytic
effects of partially purified extracts of SLS have been inhibited using trypan blue or
certain phospholipids using *in vitro* model system (Taketo and Taketo, 1966) (Ginsburg
*et al.*, 1982).

Phospholipids are natural components of cell surface membranes where the pore-
forming SLS exerts its action. The phospholipids dipalmotyl phosphatidylcholine
(DPPC) is also a major component of human lung surfactant (Rooney, 1985). The
hemolytic activity of the GBS β-hemolysin/cytolysin is inhibited by DPPC in a dose-
dependent fashion (Nizet *et al.*, 1996). In this Chapter, we examine the potential of
DPPC to inhibit cytotoxic and antiphagocytic effects of SLS, and explore its therapeutic
potential in treatment of GAS infection in the to use a phospholipid component of lung
surfactant, DPPC to inhibit the cytotoxic effects of SLS in an in-vitro cell toxicity model
and innumerate the protective effect of DPPC in our established mouse NF model.

**C2. Materials and Methods**

a. Bacterial Strains and Culture: GAS were grown in Todd-Hewitt broth (THB), on
Todd-Hewitt agar plates (THA), or on sheep blood agar plates containing 5%
defibribrinated sheeps blood in the base media of Trypticase Soy Agar (SBA). Log
phase GAS were prepared for *in vitro* and *in vivo* studies by growing the bacteria to an
OD of 0.4 measured at 600nm wavelength = \( \sim 2 \times 10^8 \) CFU/ml. These cultures were pelleted, washed with PBS, and resuspended to desired concentration either in compete media (RPMI + 2%FBS) for cell culture or in PBS for blood killing or in vivo studies.

b. Cytotoxicity assay and cell culture: A549 cells (ATCC# CCL-185), a human type II alveolar epithelial carcinoma cell line, were maintained in RPMI 1640 fortified with 10% heat inactivated fetal bovine serum (FBS). A549 cells at a density of \( \sim 2 \times 10^5 \) cells were seeded a day before the assay in a 24 well plates (Corning) with no antibiotics, media was removed and replaced with media RPMI 1640 + 2% FBS prior to adding the bacterial inoculum. For cytotoxicity assays, log-phase GAS were added at specific multiplicity of infection (MOI = 50:1 or 10:1), spun at 200 x g to place bacteria on the monolayer surface, and incubated for 2 h at 37°C in 5% CO₂.

Experimental wells contained 200 μg/ml of DPPC (Sigma) solubilized by sonication three times for 20 secs burst at 30W. Control wells added an equivalent amount of RPMI 1640. Cell viability after 2 h was measured by MTS cell toxicity assay according to manufacturer’s guidelines (Promega Cat# G5421). The human keratinocyte cell line HaCat (Boukamp et al., 1988) was propagated in RPMI media (Gibco-BRL) supplemented with 10% fetal bovine serum and seeded in 4-well chamber slides (Microtek). Newly confluent monolayers were exposed to log-phase GAS strains at MOI of 100:1 and incubated for 2h at 37°C in 5% CO₂. Experimental wells contained specified quantity of DPPC prepared in above mentioned manner. Excess bacteria were removed by gentle washing in PBS, monolayers stained with 0.04% trypan blue, then
fixed with paraformaldehyde and counterstained with eosin for quantification of stained nuclei under light microscopy as described earlier in chapter II of this dissertation and the corresponding publication (Datta et al., 2005).

c. Whole blood survival assay: Survival of bacteria in whole blood was measured utilizing a slight modified version of a method described previously in chapter II and publication of this dissertation (Datta et al., 2005). GAS were grown to log phase and diluted in PBS to obtain $10^2$ cfu in 50 µl, then mixed with 300 µl of fresh heparinized (10 U/ml) human whole blood in siliconized tubes. We then added 50 µl of 200 µg/ml final concentration of DPPC solubilized by sonic in PBS, the control well contained PBS to compensate for the volume added. The mixture was incubated at 37°C for 3 h with orbital shaking, then plated on agar media for enumeration of surviving cfu.

d. Murine model of GAS necrotizing skin infection: Experiments were performed using a well-established model (Betschel et al., 1998; Nizet et al., 2001; Timmer et al.). GAS were grown to log-phase, pelleted, washed, resuspended in PBS, and mixed 1:1 with Cytodex beads (50 mg/100 ml, Sigma). An inoculum of $10^7$ cfu GAS in 50 µl + 50 µl of bead solution and 50 µl of either solubilized DPPC (200 µg/ml final conc.) or PBS was then injected into the right flank of 4-week-old female hairless crI:SKH1(hhrhr)Br mice (n = 10 in experimental group and n=2 in control group). At 96
h, all animals were sacrificed, biopsies performed for histopathologic assessment (H & E staining), and skin, blood and spleen collected for quantitative cultures.

C3. Results and Discussion

GAS NF is a rapidly progressive infection with extensive tissue damage in association with severe toxicity, multiorgan failure and high associated morbidity and mortality (Efstratiou, 2000). The rapidly progressing bacterial infection in the fascial planes is nearly always associated with streptococcal bacteraemia (Bouvet, 2001). Prompt surgical intervention is essential to debride necrotic tissues and maximize the chances for survival and functional recovery, along with intravenous antibiotics are the cornerstones of therapy. There are reports for growing number of clinical failures to treat patients with severe invasive GAS disease with penicillin. We have shown that the SLS toxin is a critical virulence factor in the pathogenesis of GAS NF, and here test the potential of SLS inhibition as a therapeutic modality in serious GAS infection.

By weight, SLS is regarded as one of the most potent cytoxins known (Koyama, 1963), with a cytolytic spectrum that includes not only erythrocytes but other eukaryotic cells such as lymphocytes (Hryniewicz and Tagg, 1977), neutrophils (Ofek et al., 1972), tissue culture cells (Taketo and Taketo, 1966) and subcellular organelles as lysosomes.
(Bernheimer and Schwartz, 1964) and mitochondria (Keiser et al., 1964). Although trypan blue has been reported to act as a potent inhibitor of SLS (Ofek et al., 1970), there are obvious limitation of using trypan blue in the clinical setting.

Evidence exists that certain phospholipids can block the action of pore-forming cytolytic toxins. For example, the Gram-negative facultative anaerobe Actinobacillus actinomycetemcomitans elaborates a potent cytotoxin rapidly kills human leukocytes. Exogenous phospholipids with a glycerol skeleton esterified by fatty acids or positively charged liposome protects neutrophils from the action of this bacterial leukotoxin (Ginsburg et al., 1982). Similarly, the GBS hemolysin/cytolysin produces cytotoxicity as measured by lactate dehydrogenase release in A549 lung epithelial cells or by makers of apoptosis in macrophages; in each case this injury was significantly reduced in the presence of the phospholipids DPPC (Nizet et al., 1996) (Liu et al., 2004).

We first sought to assess the protective effects of phospholipids in A549 lung epithelial tissue culture cells exposed to log phase WT serotype M1 GAS bacteria elaborating SLS. For these assays, we used commercially available DPPC (Sigma). The MTS assay was used to measure viable A540 cells surviving at the end of 2 hours of GAS infection of the monolayer. Control experiments with complete media (RPMI + 2% FBS) alone or with addition of 200 μg/ml of DPPC showed no difference in baseline cell viability. Additionally, 200 μg/ml of DPPC did not affect growth of GAS in complete media (not shown). When monolayers were exposed to WT M1 GAS bacteria, cytolysis was observed, but this bacterially-induced cell injury could be
significantly mitigated by addition of DPPC to the experimental wells (Fig. III. 3). The observed protective effect of DPPC was especially evident at the higher MOI of 50 bacterial cells.

GAS NF is a cutaneous and subcutaneous disease in which the keratinocyte layer may be severely compromised and play a role in entry of the bacteria into deeper tissues (Bisno and Stevens, 1996). Thus we chose the human keratinocyte line HaCat (Boukamp et al., 1988) as a relevant cell injury model for our studies. Monolayers of HaCat cells in chamber slides were infected with GAS WT at an MOI of 100:1 for 2 h; then dead cells were stained with trypan blue and counter stained with eosin. Several high power fields were counted for the deeply blue stained nuclei, indicative of a dead cell. On an average 135 cells were counted on a single high power field and monolayers infected with SLS expressing wild type bacteria had 55 to 68% of dead cells (Fig. III. 4). On the contrary monolayers treated with DPPC were protected, with very few dead cells evident after WT GAS challenge (2-6 per high power field). The reduction of cytotoxicity approximately levels associated with the monolayers challenge with an SLS-deficient ΔsagA mutant. Such a result suggests the cytoprotective effect DPPC involves inhibiting the cytolytic action of SLS.

SLS expression is critical in the pathogenesis of GAS infection in a murine model (Betschel et al., 1998; Nizet, 2002), as I have further corroborated in my studies in chapters II and III of this dissertation (publications (Datta et al., 2005; Humar et al., 2002)). SLS expression is strongly correlated to the viability of the bacterial isolates in the skin ulcers, as well as the ability of the organism to disseminate and produce
bacteremia. Our histopathological studies have demonstrated the paradoxical feature of extensive recruitment of neutrophils with little clearance of the bacteria (Nizet, 2002). I have demonstrated that this phenomenon could be explained by the antiphagocytic effect of SLS, with the likely correlate that SLS is involved in leukocyte destruction. Utilizing the whole blood killing assay described by deMalmanche and deMartin, we repeated the assay described earlier in chapter II (publication (Datta et al., 2005)), but this time exploring the effect of DPPC addition at 200μg/ml during the experimental exposure. In this experiment, we found that WT M1 GAS proliferated rapidly (15-fold), while the WT M1 GAS exposed to human blood and DPPC grew much more slowly (4-fold) over the course of the assay (Fig. III. 5). These ex vivo results corroborate our conclusion that SLS is an important component of GAS phagocytic resistance, and that this effect is countered by addition of DPPC.

Finally, we asked whether the protective effects of DPPC against GAS mediated cytotoxicity and phagocyte resistance could be translated into therapeutic benefit in our GAS infection model. In the pilot experiment, we injected mice subcutaneously with 2x10⁷ CFU of WT GAS, along with either an intralesional dose of solublized DPPC or PBS vehicle alone. We observed that 4/10 mice infected with M1 strain alone developed obvious necrotic ulceration, compared to only 1/10 in the DPPC treatment group (Fig. III. 6A). Mice were sacrificed after 96 h for the collection of lesion (or inoculation site) biopsies and blood for quantitative cultures. DPPC treated mice had significantly lower GAS bacterial counts recovery from their skin lesion (median = 3.9 Log₁₀), that those treated with PBS control (median = 5.6 Log₁₀) (Fig. III. 6B). The
DPPC protective effect seemed to be specific for SLS neutralization, as in mice infected with the M1 ΔSLS mutant, a paradoxical mild increase in the few CFU recovered was observed upon DPPC treatment.

Our combined studies suggest there is merit in further exploring the potential therapeutic benefits of phospholipid administration as an adjunctive therapy for GAS NF, as such drugs could reduce GAS SLS-mediated cytotoxicity and phagocyte resistance, allowing more effective immune containment of the pathogen.
Fig. III. 1. Induction of TNFα mRNA transcription in bone marrow derived macrophages by wild type M1 and SLS- GAS. Total RNA from C57 wild type mice bone marrow-derived macrophages infected M1 and isogenic SLS- GAS were isolated 24h after antibiotic treatment TNF-α mRNA was quantified by RT-PCR. Non-stimulated macrophages were arbitrarily set to 1 Unit following normalization to rRNA levels (p< 0.001).
Fig.III. 2. SLS induced iNOS transcription in BM derived macrophages. Total RNA from wild type bone marrow-derived macrophages uninfected or infected with M1 and SLS- GAS for 1h and isolated 3h after antibiotic treatment. iNOS mRNA was quantified by RT-PCR. Non-infected macrophage iNOS mRNA level is set to 1, all RNA is normalized to total rRNA (p< 0.001).
Fig. III. 3. Inhibition of SLS associated cell death by the phospholipid DPPC. A549 cell monolayer treated with DPPC show increased viability of the cell infected with GAS wild type in two different MOI, compared to untreated monolayer as measured using a MTS assay.
Fig. III. 4. Cytolytic activity of SLS blocked by DPPC. SLS-mediated cell injury is blocked by DPPC treatment of human keratinocytes (HaCat) measured by trypan blue stained nuclei. (** p<0.001)
Fig. III. 5. Antiphagocytic property of SLS in GAS is overcome by addition of DPPC in whole blood. Relative survival in human whole blood of wild-type M1 strain un-treated, treated with 20mg/ml of DPPC and SLS- (sagA) mutant.
Fig. III. 6. Contribution of DPPC in protection against M1 associated necrotic skin lesion in mice. Four week old hairless crl:SKH1(hrhr)Br mice we challenged subcutaneously with 2x10^7 CFU of wild type M1 strain along with either DPPC or PBS vehicle. (A) Gross appearance of mice at 96 hrs post infection (B) GAS colony-forming units recovered from the skin lesion at 96 hrs post inoculum (p= 0.03).
REFERENCES


Chapter IV

Global transcriptional control of host phagocyte function in GAS NF by hypoxia inducible-factor 1α (HIF-1α)

A. Introduction

The eradication of invasive GAS depends on innate immunity mechanisms that act within minutes of infection. This acute inflammatory response recruits more immune cells from local blood vessels. If GAS crosses an epithelial barrier and begins to replicate in the tissues of the host, it is, in some cases, immediately recognized by macrophages that reside in those tissues. Macrophages mature continuously from circulating monocytes that leave the circulation to migrate into tissues throughout the body. The second major family of phagocytes, the neutrophils, are short-lived cells that are abundant in the blood, but are not generally present in healthy tissues. Both phagocytic cell types play a key role in innate defense, because they can recognize, ingest and destroy many pathogens, without the requirement of the adaptive immune response.

GAS colonize the skin and mucosal membranes of normal individuals, but may on occasion penetrate the epithelial barrier (or bypass a compromised epithelial barrier) to initiate a localized infection. The organisms may spread locally through tissues (e.g., cellulitis), promote abscess formation, or produce a more destructive infectious process such as NF. In each case, the response of the normal host is typically a massive infiltration of myeloid cells, primary neutrophils, to the site of infection in an attempt to
limit spread of the infectious process and eradicate the GAS (Fig. IV. 9). The scientific name of GAS (\textit{S. pyogenes} = “pus-generating streptococcus”) reflects this inflammatory process. This infiltration of neutrophils and later macrophages to the site of bacterial infection is linked with the need of these immune defense cells to respond to the tissue microenvironment. In the collaboration of our laboratory with that of Dr. Randall Johnson in the UCSD Division of Biological Sciences, have found evidence that the hypoxia-inducible transcription factor HIF-1, and its target genes, are essential for inflammation and for an effective response to GAS challenge.

In tissues injured by trauma or infection, microenvironmental conditions are characterized by low oxygen and nutrient levels (Saadi et al., 2002; Schor et al., 2000). In healthy tissues, the oxygen tension is 20 - 70 mmHg (i.e., 2.5 - 9% oxygen), whereas poor perfusion of infected tissues can drive oxygen tensions to < 10 mm Hg (i.e., less than 1% oxygen) (Lewis et al., 1999). In response to GAS infection, neutrophils cross the vessel wall, migrating into the tissue. The disruption of the blood supply to damaged or inflamed tissues results in low oxygen tension. Low oxygen levels have been described in virtually every site of extensive inflammation (Korhonen, 2000; Najafipour and Ferrell, 1995; Ott, 1987; Sawyer et al., 1991; Silver, 1975; Simmen et al., 1994), including sites of cutaneous inflammation, such as skin infections and wounds (Arnold et al., 1987) and necrotic tissue foci (Denko et al., 2000; Hockel and Vaupel, 2001).

The effectiveness of neutrophils and macrophages in innate immunity against GAS reflects an array of specialized cellular functions. First, myeloid cells must
recognize chemotactic signals to engage endothelial receptors, undergo diapedesis, and exit the microcirculation and migrate to the tissues. Recognition of GAS, enhanced by opsonins such as complement and antibody, leads to uptake of the bacterium in the phagosome which later undergoes acidification. The phagosome fuses with one or more lysosomes to generate phagolysosomes, in which the lysosomal contents are released to kill the GAS. The production of reactive oxygen species, such as hydrogen peroxide and singlet oxygen that kill GAS, especially since it lacks neutralizing catalase activity. The reactive oxygen species are generated by lysosomal NADPH oxidases in a process known as the respiratory burst. Myeloid cells also possess granules that contain antimicrobial peptides (e.g. cathelicidins) and proteases (e.g. elastase, cathepsin) that can be released to enhance GAS killing. The myeloid cells arriving at the infection site also release pro-inflammatory cytokines (e.g. IL-8, TNF-α) to amplify the immune response and recruit additional neutrophils. Vasoactive factors (e.g. VEGF, and also NO) are also released, to increase microvascular permeability and allow extravasation of immune cells in the infected tissues. These multiple functions of the myeloid cell in innate immune defense require substantial energy generation.

Neutrophil motility, chemotaxis and aggregation are all fueled by extracellular glucose uptake (Weisdorf et al., 1982). This reliance on the glycolytic pathway is thus a key feature of the extravascular existence of these myeloid cells. HIF-1 plays a role most aspects of hypoxia-induced gene expression, and is essential for hypoxia-induced increases in glycolysis in normal or diseased tissue (Seagroves et al., 2001)(Semenza, 2001). HIF-1 is a transcription factor whose expression is regulated by oxygen at the
protein level. HIF-1 is heterodimer made up of two helix-loop-helix proteins; HIF-1α, which is the oxygen-responsive component, and the constitutively expressed HIF-1β. HIF-1α is only detected under low oxygen concentrations, and is rapidly degraded by the ubiquitin-proteasome pathway under ambient conditions (Semenza, 2001). This process of degradation under normoxia is directed by its interaction with the von Hippel-Lindau (VHL) tumor suppressor protein. In response to hypoxia, HIF-1α protein accumulates and translocates to the nucleus to bind the constitutively expressed HIF-1β. The heterodimer then binds hypoxic response elements (HREs) of target gene regulatory sequences (Fig. IV, 10). Some of these direct target genes include glucose transporters, the glycolytic enzymes, erythropoietin and angiogenic factor vascular endothelial growth factor (VEGF).

Employing conditional gene targeting in myeloid cells, the Johnson laboratory performed a comprehensive analysis of the hypoxic response in myeloid cell-mediated inflammation (Cramer et al., 2003). Targeted deletions of the HIF-1α and VHL genes, via crosses into a background of cre expression driven by the lysozyme M promoter (lysMere) (Clausen et al., 1999), allowed specific deletion of either factor in the myeloid lineage. The neutrophil and macrophage-specific knockout mice were used to determine the contribution of HIF-1α and vHL to myeloid cell inflammatory responses. These deletions did not change numbers of circulating monocytes or neutrophils (Cramer et al., 2003). HIF-1α was found to be a regulator of VEGF expression in macrophages and eliminated the hypoxia-induced augmentation of the TNF-α response to lipopolysaccharide (Hempel et al., 1996; VanOtteren et al., 1995). HIF-1α
null macrophage release of lactate was significantly lower than WT cells, and failed to be induced by LPS stimulation. In contrast, vHL null cells showed significantly increased lactate production, indicating that the loss of vHL results in increased glycolytic activity of macrophages under both basal and LPS-stimulated conditions (Cramer et al., 2003). ATP levels in isolated HIF-1α null macrophages and neutrophils were found to be dramatically reduced, down to levels known to impair function in studies with standard glycolysis inhibitors (Boxer et al., 1977).

Two dramatic phenotypes were observed in the initial report of HIF-1α role in myeloid cell inflammation. First, the mice failed to produce a skin irritation in response when the chemical SDS was applied to the skin, indicating a clear defect in producing an inflammatory response in these tissue (Fig. IV. 11). Second, a defect in the ability of macrophages to kill group B Streptococcus following phagocytotic uptake was also documented (Fig. IV. 12). These findings suggested that HIF-1α may serve as a master regulator of the inflammatory and innate immune functions of neutrophils and macrophages.

In this Chapter, I report studies in which I was the principal member of the Nizet laboratory conducting a thorough experimental analysis of myeloid cell bactericidal activity in direct collaboration with a postdoctoral fellow of the Johnson laboratory, Carole Peyssonnaux. I participated directly in all studies involving bacterial challenges in the isolated WT, HIF-1α-null and vHL-null neutrophils and macrophages and neutrophils and led the in vivo challenge experiments in the murine model of GAS
necrotizing fasciitis. These studies comprise the included manuscript:

“HIF-1\(\alpha\) expression regulates the bactericidal capacity of phagocytes.”
HIF-1α expression regulates the bactericidal capacity of phagocytes


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Hypoxia is a characteristic feature of the tissue microenvironment during bacterial infection. Here we report on our use of conditional gene targeting to examine the contribution of hypoxia-inducible factor 1, α subunit (HIF-1α) to myeloid cell innate immune function. HIF-1α was induced by bacterial infection, even under normoxia, and regulated the production of key immune effector molecules, including granule proteases, antimicrobial peptides, nitric oxide, and TNF-α. Mice lacking HIF-1α in their myeloid cell lineage showed decreased bactericidal activity and failed to restrict systemic spread of infection from an initial tissue focus. Conversely, activation of the HIF-1α pathway through deletion of von Hippel-Lindau tumor-suppressor protein or pharmacologic inducers supported myeloid cell production of defense factors and improved bactericidal capacity. HIF-1α control of myeloid cell activity in infected tissues could represent a novel therapeutic target for enhancing host defense.

Introduction
The eradication of invading microorganisms depends initially on innate immune mechanisms that preexist in all individuals and act within minutes of infection. Phagocytic cell types, including macrophages and neutrophils, play a key role in innate immunity because they can recognize, ingest, and destroy many pathogens without the aid of an adaptive immune response. The effectiveness of myeloid cells in innate defense reflects their capacity to function in low-oxygen environments. Whereas in healthy tissues oxygen tension is generally 20–70 mm Hg (i.e., 2.5–9% oxygen), much lower levels (<1% oxygen) have been described in wounds and necrotic tissue foci (1–3).

The adaptive response of mammalian cells to the stress of oxygen depletion is coordinated by the action of hypoxia-inducible transcription factor 1 (HIF-1). HIF-1 is a heterodimer whose expression is regulated by oxygen at the protein level. The protein stability of the α subunit (HIF-1α) is regulated by a family of prolyl hydroxylases. This process is directed by the interaction of HIF-1α with the von Hippel-Lindau tumor-suppressor protein (VHL). Under hypoxia, prolyl hydroxylase activity is inhibited, and HIF-1α accumulates and translocates into the nucleus, where it binds to HIF-1β, constitutively expressed. The heterodimer HIF-1 binds to the hypoxia response elements (HREs) of target gene regulatory sequences, resulting in the transcription of genes implicated in the control of metabolism and angiogenesis as well as apoptosis and cellular stress (4). Some of these direct target genes include glucose transporters, glycolytic enzymes, erythropoietin, and the angiogenic factor VEGF. Two additional HIF subunits have subsequently been cloned and named HIF-2α (5–7) and HIF-3 (8), but their regulation is less well understood.

Confirmation that HIF-1α was expressed in activated macrophages (9, 10) led us to explore the function of this transcription factor in the myeloid cell lineage. Employing conditional gene targeting, we recently showed that HIF-1α control of the metabolic shift to glycolysis plays an important role in myeloid cell–mediated inflammatory responses (11). These studies also provided preliminary in vitro evidence that deletion of HIF-1α could impair myeloid bactericidal activity. The effectiveness of neutrophils and macrophages in innate antibacterial defense reflects a diverse array of highly specialized cellular functions including phagocytic uptake of the bacterium, production of reactive oxygen species, activation of iNOS, and release of antimicrobial peptides (e.g., cathelicidins, defensins) and granule proteases (e.g., elastase, cathepsin). Here we perform a detailed analysis of the underlying mechanisms by which HIF-1α transcriptional control pathways contribute to the antibacterial function of myeloid cells, and for the first time, to our knowledge, determine the requirement of HIF-1α expression for myeloid cell–mediated innate immune defense in vivo. Our results indicate a pivotal role for HIF-1α in myeloid cell biology under both hypoxia and normoxia and suggest that this transcription factor may represent a unique therapeutic target for boosting immune defense function in tissues compromised by bacterial infection.

Results
Bacteria induce HIF-1α expression. Invasive pyogenic bacterial skin and soft tissue infections generate localized tissue ischemia, thrombosis, and necrosis and represent a formidable test of the adaptiveness of neutrophils and macrophages in hypoxic microenvironments. In this regard, a strain of the Gram-positive pathogen group A Streptococcus (GAS), isolated from a patient with necrotizing fasciitis (flesh-eating disease), was chosen as the primary
model organism for most in vitro and in vivo challenges. We found that expression of HIF-1α was increased 4-fold in WT mouse macrophages following exposure to GAS under normoxic conditions (Figure 1A). Indeed, GAS represented a more potent stimulus for HIF-1α induction than hypoxia itself. The phenomenon of bacterial induction of HIF-1α under normoxia was also observed with additional Gram-positive (methicillin-resistant Staphylococcus aureus, hereafter S. aureus) and Gram-negative (Pseudomonas aeruginosa, hereafter P. aeruginosa, and Salmonella typhimurium) bacterial species of medical importance (Figure 1A).

We next evaluated whether the induction of HIF-1α protein by GAS corresponded to an increase in HIF-1α transcriptional gene activation. We measured HIF-1α-dependant transcription in macrophages derived from HRE-luciferase transgenic mice, which contain a luciferase reporter gene driven by 6 consecutive specific HRE sequences. As shown in Figure 1B, a 3-fold increase in luciferase reporter activity was reached after incubating the macrophages for 18 hours in 1% oxygen or in the presence of known pharmacologic inducers of HIF-1α, including desferrioxamine mesylate, cobalt chloride (CoCl2), and L-mimosine (L-Mim). Incubation of the reported macrophages with live or heat-killed GAS bacteria at normoxia stimulated luciferase activity to levels comparable to or greater than those of hypoxia (Figure 1B).

HIF-1α regulates bactericidal capacity of myeloid cells. To assess the functional consequences of HIF-1α activation, we used an anti-biotic protection assay to calculate intracellular killing of GAS by WT macrophages compared with killing by those derived from the bone marrow of HIF-1α-null mice. Here, targeted deletion of the HIF-1α gene has been created via crosses into a background of cre expression driven by the lysozyme M promoter (lysMc), allowing specific deletion of the transcription factor in the myeloid lineage (11). As shown in Figure 2A, intracellular killing of GAS by WT macrophages was increased under hypoxia, providing initial indication that HIF-1α may be involved in the bactericidal process. This result was especially notable because the facultative GAS bacteria lack oxidative phosphorylation and grow more rapidly under anaerobiosis (12). We found that, compared to WT cells, macrophages from HIF-1α-null mice showed a 2-fold decrease in GAS intracellular killing under normoxia and a 3-fold decrease in GAS intracellular killing under hypoxia (Figure 2A). Time-course studies showed that the killing defect observed in HIF-1α-null macrophages increased over time, such that 15-fold more viable bacteria were present within HIF-1α-deleted cells by the last time point 120 minutes (Figure 2B). Macrophage killing of the Gram-negative bacterium P. aeruginosa was likewise impaired upon deletion of HIF-1α (Figure 2B).

As a complementary analysis of the linkage of myeloid cell bactericidal functions with HIF-1α transcriptional control, we explored the effects of increased HIF-1α activity on bacterial killing by using macrophages derived from vHL-null mice. vHL is a key regulator of HIF-1α turnover; these mice have constitutively high levels of HIF-1 activity in the deleted cell population (11). We found that vHL-null macrophages showed increased intracellular killing of GAS and P. aeruginosa compared with WT cells across multiple time points (Figure 2C). Similar differences were observed in macrophage bactericidal assays that omitted antibiotics and instead employed vigorous washing to quantify total surviving cell-associated GAS or P. aeruginosa (not shown). Macrophage populations isolated from WT, HIF-1α-null, and vHL-null mice both included more than 99.8% differentiated macrophages by flow cytometric analysis, and Trypan blue staining showed similar levels of macrophage viability (98–99%) throughout the GAS-killing assays (not shown). These controls suggest that there exists an intrinsic defect in the bactericidal activity of HIF-1α-null cells that cannot be attributed to differences in the purity or viability of the explanted cell populations.

Finally, we treated WT macrophages with a number of known pharmacologic inducers of HIF-1α that each act directly or indirectly to inhibit prolyl hydroxylase targeting of HIF-1α for ubiquitination. These included the iron chelator desferrioxamine, CoCl2, L-Mim, and 3-hydroxy-1,2-dimethyl-4-(H)pyridone (OH-pyridone) (13). The addition of each of these agents increased intracellular killing of GAS by WT macrophages (Figure 2D). Assays were performed using a concentration of each agonist and exposure time that did not affect bacterial viability (not shown).

Myeloid cell HIF-1α production is important for control of GAS infection in vivo. We chose an animal infection model of GAS-induced necrotizing soft tissue infection for directly testing myeloid cell bactericidal function in vivo. We introduced the GAS inoculum subcutaneously into a shaved area on the flank of WT and HIF-1α-null male littermates and followed progression of the infection over 96 hours. We found that mice with a tissue-specific deletion of HIF-1α in macrophage and neutrophils developed significantly larger necrotic skin lesions and experienced greater weight loss than WT mice (Figure 3, A and B). Representative gross appearance of the necrotic lesions in WT and HIF-1α myeloid-null mice is shown in Figure 3C. We next asked
whether myeloid cell production of HIF-1α was important in limiting the ability of GAS to replicate within the necrotic skin tissues and to disseminate from the initial focus of infection into the bloodstream and systemic organs. Mice were sacrificed at 96 hours after inoculation and quantitative bacterial cultures performed on the skin ulcer (or site of inoculation if no ulcer developed), blood, and spleen (Figure 3D). Approximately 1,660-fold greater quantities of GAS were present in the skin biopsies of HIF-1α-null mice compared with those of WT mice. Similarly, 27-fold (blood) or 85-fold (spleen) more bacteria were isolated in systemic cultures from HIF-1α-null mice compared with WT mice. Our findings indicate that the presence of HIF-1α transcriptional control in neutrophils and macrophages is important in limiting the extent of necrotic tissue damage and preventing systemic spread of bacterial infection.

HIF-1α is not critical for neutrophil endothelial transcytosis or oxidative burst function. We next began a series of experiments to probe the potential cellular and molecular mechanisms through which HIF-1α may support myeloid cell functional killing capacity in vitro and in vivo. Although histopathologic examination of the biopsies from the necrotic ulcers generated by GAS revealed clear tissue ischemia by HypoxyProbe (Figure 4A), the observed immune defect of HIF-1α-null animals did not appear to reflect impaired phagocyte recruitment, since similar numbers of neutrophils were observed on immunostaining of the skin tissue of WT compared with that of HIF-1α-null mice at 6, 12, and 24 hours after infection (Figure 4B). The latter finding differed qualitatively from our previous study, in which decreased neutrophil infiltration was seen in skin tissue of HIF-1α after chemical irritation with the phorbol ester tetradecanoyl phorbol acetate (11), and from the prediction that might be derived from HIF-1α control of β2 integrin expression (14). We speculate that the stimulus to neutrophil migration elicted by bacterial infection is perhaps stronger and more complex (i.e., involving more pathways) than that of chemical irritation such that the any contribution of HIF-1α may be muted in comparison to its effects on bacterial killing. To explore further whether the migratory capacity of WT and HIF-1α-null neutrophils toward a bacterial stimulus was indeed unaffected, we measured the rate of transcytosis across murine endothelial cell monolayers following stimulation by GAS or the bacteria-derived chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). In these assays, we also found no significant difference in transendothelial migration between the activated WT, HIF-1α-null, and vHL-null murine neutrophils (Figure 4C).

The production of reactive oxygen metabolites generated by lysoosomal NADPH oxidases in a process known as the respiratory
burst is a major mechanism of bacterial killing. However, circulating neutrophils derived from HIF-1α-deficient or vHL-deficient mice were similar to WT neutrophils in oxidative burst activity (Figure 4D). Thus, the defect in innate immunity to GAS infection observed in HIF-1α myeloid–null mice could not be attributed to impairment in oxidative burst function.

Production of granule proteases and antimicrobial peptides is regulated by HIF-1α. Granule proteases are increasingly recognized as an important component of myeloid cell antimicrobial activity. Neutrophil elastase (NE) and cathepsin G are abundant serine proteases concentrated in the granules that are primarily destined to fuse to phagosomes and form phagolysosomes. Gene targeting of elastase in mice has directly supported a role of NE in host innate immune defense (15), and accumulating evidence suggests a similar role for cathepsin G (16–18). Patients with Chediak-Higashi syndrome lack NE and suffer recurrent bacterial infections. To determine whether HIF-1α has an impact on neutrophil production of granule proteases, we measured NE and cathepsin G activity in WT, HIF-1α–null, and vHL–null blood neutrophils. Protease activity was measured using a synthetic peptide substrate containing recognition sites for each molecule to allow either fluorometric (NE) (Figure 5A) or spectrophotometric (cathepsin G) detection (19) (Figure 5B). HIF-1α–null neutrophils showed decreased enzymatic activity of each granule protease compared with WT neutrophils while vHL–null neutrophils exhibited increased protease activity. Mixing experiments with WT and HIF-1α–null macrophages excluded the possibility that HIF-1α–null neutrophils produce a greater amount of an (unknown) inhibitor rather than less of the granule proteases (Figure 5, A and B).

The production of proteases by neutrophils may exert direct antimicrobial effects or, alternatively, may serve to activate cationic antimicrobial peptides from their inactive precursor forms (20, 21). An important component of innate immune defense in mammals is the cathelicidin family of antimicrobial peptides (22). These gene-encoded “natural antibiotics” exhibit broad-spectrum antimicrobial activity and are produced by several mammalian species on epithelial surfaces and within the granules of phagocytic cells. Proteolytic cleavage of an inactive precursor form to release the mature C-terminal antimicrobial peptide is accomplished by proteases, such as elastase, upon degradation of activated neutrophils (23). Mice have a single cathelicidin, cathelicidin-related antimicrobial peptide (CRAMP), which closely resembles the single human cathelicidin (LL-37). Importantly, we demonstrated in earlier experiments using the murine model of necrotizing skin infection that endogenous production of CRAMP was essential for mammalian innate immunity to GAS (24). We performed experiments to identify whether production or activation of CRAMP was under HIF-1α control. Lytesates from WT, HIF-1α–null, and vHL–null peritoneal neutrophils were analyzed by SDS-PAGE and immunoblotted with a rabbit anti-mouse CRAMP antibody against the CRAMP mature peptide. HIF-1α deletion led to a dramatic reduction of the active mature form of cathelicidin compared with WT neutrophils while CRAMP was expressed at higher levels in vHL-deficient neutrophils (Figure 5C).

Regulation of cathelicidin expression occurred at least in part at the mRNA level, as CRAMP transcript levels are reduced by 80% in HIF-1α–null macrophages, and conversely increased with loss of vHL (Figure 5D). As would be expected, CRAMP mRNA was also increased by exposure of the neutrophils to hypoxia (Figure 5D).
Thus, the production and activation of cathelicidin antimicrobial peptides represents an additional myeloid cell killing mechanism that is affected by alterations in the HIF-1α pathway.

HIF-1α is a principal regulator of NO production in response to bacterial infection. NO is known to exert antimicrobial properties against a variety of bacterial species (23). Nitric oxide is enzymatically produced by NOS through the oxidation of arginine, and mice deficient in iNOS are more susceptible to bacterial infection (24, 27). It has been well documented that HIF-1α is a transcriptional activator of iNOS expression (28–30), but no studies have examined this link in the context of bacterial infection. Here we found that exposure of macrophages to GAS increased iNOS mRNA production approximately 250-fold (Figure 6A). Deletion of HIF-1α resulted in an approximately 70% reduction in iNOS gene transcription, while deletion of vHL led to a marked increase in iNOS mRNA levels (Figure 6A). Measurement of nitrite in cell culture supernatants confirmed that the observed differences in iNOS induction translated directly to differences in NO production (Figure 6B).

Addition of the NOS inhibitor 1-amino-2-hydroxyguanidine, p-toluenesulfate (AG) significantly inhibited the production of NO in response to GAS (Figure 6B). WT macrophages treated with L-Mim, a pharmacological inducer of HIF-1α, showed greatly increased expression of iNOS mRNA, but this increased expression was very low in HIF-1α-null cells (Figure 6C), confirming a dependency of the observed effect on the presence of the transcription factor. These experiments indicate that augmentation of iNOS expression and subsequent bacterial killing (Figure 2C) can be pharmacologically induced through increased HIF-1α expression.

To establish the functional importance of HIF-1α–induced iNOS expression and NO production, we performed macrophage bactericidal assays in the presence or absence of AG. Figure 6D shows that AG inhibited the bactericidal activity of WT macrophages but did not further suppress the poor bactericidal activity of HIF-1α-null macrophages. We found similar results using the iNOS-specific inhibitor 1400W (not shown). It has recently been demonstrated that NO, as well as certain reactive oxygen species, cytokines, and growth factors, can contribute to stability regulation of HIF-1α and HIF-1 transactiva-

Figure 4
HIF-1α is not critical for neutrophil endothelial transcytosis or oxidative burst function. (A) Hypoxia is present in lesions generated by GAS infection. Immunostaining for hypoxic markers in WT mouse skin upon GAS infection. Magnification, ×100 (top); ×200 (bottom). The control corresponds to the omission of primary antibody. (B) Similar numbers of neutrophils in WT and HIF-1α–null mouse skin tissue observed by Immunostaining at 6, 12, and 24 hours after infection. Magnification, ×100. (C) Migratory capacity of activated neutrophils across endothelium is not affected by the deletion of HIF-1α. Count of neutrophils transcytosing pulmonary endothelial monolayer toward GAS or fMLP stimulus is shown. (D) HIF-1α activity does not affect oxidative burst capacity. Flow cytometry of leukocytes derived from WT (squares), HIF-1α–null (triangles) and vHL–null (inverted triangles) mice. Oxidative burst capacity as measured by fluorescence before (0 seconds) and after the addition of a reagent designed to stimulate leukocyte phagocytic and oxidative activity as described in Methods. Data are representative of the results obtained for 4 individuals per genotype.
TNF-α protein expression was similar in WT, HIF-1α, and vHL-null macrophages, loss of HIF-1α also strongly depressed the rapid secretion of TNF-α protein in response to GAS (Figure 7B). As NO is markedly induced under GAS stimulation, TNF-α induction by GAS may rely on HIF-1α-dependent NO production. ELISA for secreted TNF-α demonstrated reduced amounts of TNF-α protein in conditioned supernatants of WT, HIF-1α, and vHL-deficient macrophages in the presence of the iNOS inhibitor AG (Figure 7B). This finding indicates that NO production, acting in a HIF-1α-controlled manner, contributes significantly to the macrophage TNF-α response to bacterial infection.

Discussion

Our studies use conditional gene targeting in the myeloid cell lineage to demonstrate that HIF-1α transcriptional regulation plays an important role in innate immunity to bacterial infection. Activation of HIF-1α under hypoxia enhances bactericidal activity, and HIF-1α pathways are responsive to bacterial stimulation even under normoxia. While certain myeloid cell functions, including endothelial transmigration and respiratory burst activation, appear to be independent of HIF-1α control, the transcription factor is involved directly or indirectly in the regulation of specific immune functions including NO, granule proteases (cathpsin G, NE), and cathelicidin antimicrobial peptides. The marked reduction of granule protease and cathelicidin expression in HIF-1α-deficient neutrophils correlates with diminished bactericidal activity in vitro and failure to control infection in vivo, lending support to recent studies uncovering a key role for these neutrophil effectors in malignant innate immunity (15, 24).

Successful control of infection in the peripheral tissues requires that host myeloid phagocyte cells function effectively in hypoxic environments. The challenge to immune defense is made more critical when the microbial toxins or local edema damage host cells and the vascular supply of oxygen to the tissues becomes further compromised. The placement of essential microbicidal killing functions of myeloid cells under regulation of HIF-1α therefore represents an elegant controlled-response system (Figure 8). Bactericidal mechanisms can be maintained in an "off" state while the myeloid cells circulate in the oxygen-rich bloodstream and then be activated in response to the declining oxygen gradient encountered upon diapedesis and entry of the phagocytes into the infected tissues. Additional, more potent stimulation of the HIF-1α transcriptional pathway is then provided by direct encounter with the bacteria (Figure 1A). A regulatory mechanism by which HIF-1α targets genes involved in microbial killing ensures that the corresponding inflammatory mediators are expressed preferentially in tissue foci of infection but not in healthy tissues where inflammatory damage might otherwise harm host cells.

Our experiments also reveal that NO production is a myeloid cell-killing mechanism principally regulated by HIF-1α during bacterial infection. Further, we suggest that NO is likely to play a key role in the amplification of the inflammatory response through stimulation of TNF-α release. Although the effects of inflammatory cytokines on regulating NO production have been extensively studied (38–40), the reverse relationship pertaining to the effect of NO on cytokines remains controversial (41–44). A recent study demonstrated that suppression of NO could inhibit IPS-induced TNF-α and IL-1 release and pinpointed such modulation at the transcriptional level (45). We find here that macrophage production of TNF-α is dependent on NO levels controlled in turn by HIF-1α transcriptional regulation of iNOS.

Recent data has established that HIF-1α is subjected to stability regulation by soluble intracellular messengers, such as NO and TNF-α (33, 34). With such processes at play, one can speculate that HIF-1α is situated at the center of an amplification loop mechanism for innate immune activation: stimulation of HIF-1α by oxygen depletion and bacterial exposure induces the production of NO and TNF-α, which function not only to generate inflammation and control bacterial proliferation but also as regulatory molecules to further stabilize HIF-1α in myeloid cells recruited to the infectious focus.

The relative contributions of HIF-1 and HIF-2 to the regulation of gene expression in hypoxic macrophages is still under debate.
Detectable levels of HIF-2α, but not HIF-1α, have been found in a human prononocytic cell line following hypoxic induction in vitro and in tumor-associated macrophages (46, 47). In contrast, immunoreactive HIF-1α has been detected in human macrophages in the hypoxic synovia of arthritic human joints (10), and human macrophages accumulate higher levels of HIF-1 than of HIF-2 when exposed to tumor-specific levels of hypoxia in vitro (9). Our present results also clearly support a specific and independent action of HIF-1α. Taken together, these findings suggest that HIF-1α may be the major hypoxia-inducible transcription factor in macrophages.

In summary, our results demonstrate that HIF-1α not only helps myeloid cells shift to glycolytic metabolism (11) but also functions in coordinating a proper innate immune response for bacterial killing in hypoxic microenvironments. The in vitro studies confirm that the HIF-1α pathway can play a critical role in controlling proliferation of a bacterial pathogen in compromised tissues. Recent commentaries have suggested that downregulation of HIF-1α could have a therapeutic effect in disease states characterized by chronic inflammation (48, 49). We now have shown that medically important bacterial species such as GAS, methicillin-resistant S. aureus (MRSA), P. aeruginosa, and Salmonella species can trigger HIF-1α expression. Thus, the present studies suggest that rational design of pharmaceutical HIF-1α agonists (or vHIF antagonists) to boost myeloid cell microbicidal activity may likewise represent a novel approach for adjunctive therapy of complicated infections due to antibiotic-resistant pathogens or compromised host immunity.

Methods

All procedures involving animals were reviewed by the University of California San Diego Animal Care Committee, which serves to ensure that all federal guidelines concerning animal experimentation are met. Harvest of neutrophils, macrophages, and blood leukocytes. Neutrophils were either isolated from the peritoneal cavity 3 hours after injection of thioglycollate as previously described (11, 50) or derived from bone marrow as described (51). To isolate BM-derived macrophages, the marrow of femurs and tibias of WT, HIF-1α myeloid-null, or vHIL myeloid-null mice were collected. Cells were plated in DMEM supplemented with 10% heat-inactivated FBS and 50% conditioned medium (7-day supernatant of fibroblasts from cell line L-929 stably transfected with an M-CSF expression vector). Mature adherent BM cells were harvested by gentle scraping after 7 days in culture. To isolate blood leukocytes, 200–500 μl of whole blood was collected by retroorbital bleed into cold EDTA-coated capillary tubes (Terumo Medical Corp.). Cells were centrifuged, erythrocytes were lysed using ACK RBS lysis buffer (0.15 M NaCl, 10.0 mM KHCO₃, 0.1 mM EDTA), and unlysed cells were washed once with 1 ml PBS 1% BSA.

Bacterial strains and media. GAS strain 544B is an M1 serotype isolate from a patient with necrotizing fasciitis and toxic shock syndrome (52). Additional bacterial strains were obtained from the ATCC Bacteriology collection, specifically methicillin-resistant S. aureus (ATCC 35351, designation 328), S. typhimurium (ATCC 1311), and P. aeruginosa (ATCC 27853, designation Boston 41591): GAS was propagated in Todd-Hewitt broth (THB) (Difco, BD Diagnostics) and other strains in Luria-Bertani broth.
Bacterial killing assay. GAS were grown to logarithmic phase in THB to OD600 = 1 × 10^6 cfu/mL. Bacteria were added to macrophages at an MOI of 2.5 bacteria/cell and intracellular killing assessed using an antibiotic protection assay (11, 53) or, alternatively, total cell-associated bacteria measured by vigorous washing with PBS × 3 to remove nonadherent bacteria. At the end of the assay, total cell lysate was plated on THB agar for enumeration of CFU. Comparable studies were performed with P. aeruginosa at an MOI of 25. To assess macrophage viability, the monolayers were washed with PBS and incubated with 0.04% Trypan blue for 10 minutes at 37°C. As specified in the Figure 2 legend and in Results, macrophages were preincubated with L-Mimosine (800 µM), OH-phenol (150 µM), desferrioxamine methylate (100 µM), or CoCl2 (100 µM) for 5 hours prior to the killing assay; each drug level was known to be sufficient for HIF-1α induction (13). Absence of bacterial inhibition was tested by incubating the drugs at the above concentrations with GAS (~10^8) at 37°C for 1–24 hours.

Mouse model of GAS infection. An established model of GAS subcutaneous infection was adapted for our studies (24, 54). Briefly, 100 µl of a midlogarithmic phase (~10^8 cfu) of GAS was mixed with an equal volume of sterile Cytoxan (Sigma-Aldrich) and injected subcutaneously into a shared area on the flank of 5- to 8-week-old male C3H/HeJ mice. Mice were weighed daily and monitored for development of neotenic skin lesions. After 96 hours, skin lesions, spleen, and blood (via retroorbital bleeding) were collected and homogenized in 1:1 mg/mL PBS. Serial dilutions of the mixture were plated on THB agar plates for enumeration of CFUs.

Immunochemistry. Lesions were processed, embedded in paraffin, and routine sections (5 µm) cut. Immunohistochemistry was performed with an antibody specific for neutrophils (purchased from mouse anti-neutrophil mAb, Accurate Chemical & Scientific Corp.) as described (55). To assess development of hypoxic regions within the lesions, mice were injected intraperitoneally with 60 mg/kg (weight/volume in PBS) primidone (Hydroxyprobe-1, Natural Pharmaceuticals International Inc.) 2 hours prior to sacrifice. Immunohistochemistry was performed with Hydroxyprobe-1 mouse monoclonal antibody as reported (56).

Reverse transcription and real-time quantitative PCR. First-strand synthesis was obtained from 1 µg of total RNA isolated with Trizol reagent ( Molecular Research Center Inc.) by the SuperScript system (Invitrogen Corp.), employing random primers. For real-time PCR (RT-PCR) analyses, cDNAs were diluted to a final concentration of 10 ng/µl and amplified in a TaqMan Universal Master Mix, SYBR Green (Applied Biosystems). cDNA (50 ng) was used as a template to determine the relative amount of mRNA by RT-PCR in triplicate (ABI PRISM 7700 Sequence Detection System; Applied Biosystems), using specific primers with the following sequences: iNos forward 5’-ACCCCTAAGGCTCACAAATAAGGC-3’; iNos reverse 5’-TGATTCCCTACATACTGTTAGC-3’; TNFα forward 5’-CCATTCCCTAGGATCTGTATTTC-3’; TNFα reverse 5’-AGGTAGGAGGGCTCTGAGATCTTATC-3’; TNFα probe 5’-6FAMJAGGTGCTGAGGTTGCTGCTTGCTCATTACATGTA-3’; CRAMP forward 5’-CTTCACCCAGGTCCTCTTAGCACA-3’; CRAMP reverse 5’-TCCAGGCCTCCAGGAGGTA-3’; elastase forward 5’-TGCCACACCTCTCCGAG-
Nitr  determination. The concentration of nitrate (NO$_3^-$), the stable oxidized derivative of NO, was determined in 100 μl aliquots of cell culture supernatants transferred to 96-well plates. Essentially, 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethenediamine dihydrochloride, 2% H$_3$PO$_4$) was added per well, and the absorbances were measured at 540 nm in a microplate reader. Sodium nitrite diluted in culture medium was used as standard.

*Eluate and cation G assays.* For eluate measurement, 100 μl of 0.2M Tris-HCl (pH 8.5) containing 1M NaCl was mixed with 50 μg of blood leukocytes lysed in HTAB buffer containing 0.1M Tris-Cl, pH 7.6, 0.15 M NaCl, and 0.5% deoxycholate. The mixture was then centrifuged at 10000 rpm for 10 min to collect the supernatant. Fifty ng of protein or nuclear extracts were loaded on a 10% Tris-tricine gel in an MES buffer (Tris-glycine buffer) or 3-8% Tris-tricine gel in a Tris-acetate buffer (Invitrogen Corp.) for CRAMP and HIF-1α Western blot respectively.

Proteins were transferred to a nitrocellulose membrane; the membrane was blocked in 5% nonfat milk in 0.2% Tris-buffer and then incubated in primary Ab-diluted in 1% nonfat milk. The primary Ab used were rabbit anti-mouse CRAMP against the CRAMP mature peptide and rabbit anti-mouse HIF-1α (Cayman Chemical Co.). The secondary Ab was peroxidase-conjugated goat anti-rabbit (DAKO Corp.). Immunoreactive proteins were detected using the ECL chemiluminescent system (Amersham Biosciences).

*Reporte assay.* Macroorganisms were derived from the marrow of femurs and tibia of transgenic HRE-luciferase mice as described above. The luciferase reporter gene is driven by 5 specific HRE sequences. Cells were incubated with GAS or heat-inactivated GAS for 18 hours.

Luciferase activity of cell lysates was performed by using the Bright-Glo

Luciferase Assay Kit (Promega Corp.). Luciferase activities were measured using a luminometer.

Oxidative burst assay. Isolated total blood leukocytes were resuspended at 4°C in approximately 200 μl endotoxin- and pyrogen-free PBS, lacking Ca$^{++}$ and Mg$^{++}$ but containing 5 mM glucose. Immediately before the oxidative burst assay, 200 μl of PBS at 37°C containing 1.5 mM Mg$^{++}$ and 1.0 mM Ca$^{++}$ were added to the cell suspension. Oxidative burst activity was measured by using the Fc-OnyBURST Green assay reagent (Invitrogen Corp.) according to the manufacturer’s instructions.

Endothelial cell transmigration assay. Thiotoglobin-stimulated neutrophils were added to the upper chamber of a Transwell membrane (Corning HTS) coated with a primary mouse pulmonary endothelial monolayer. The chemokine (rMCP-1 8 μg/ml or GAS [Mn 10]) was added to the lower well. The number of neutrophils migrating to the lower chamber was counted after 1 hour of incubation at 37°C.

*Reagents.* AG and 1400W were purchased from EMD Biosciences. L-Mim, OFF-pyridone, desferrioxamine mesylate, and CoCl$_2$ were purchased from Sigma-Aldrich.

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Chapter IV B, in full, is a reprint of the material as it appears in Peyssonaux C, Datta V, Cramer T, Doedens A, Theodorakis E, Gallo R, Hurtado-Ziola N, Nizet V, Johnson RS. HIF-1alpha expression regulates the bactericidal capacity of phagocytes.

Fig.IV. 9. **Migration of myeloid cells to hypoxic microenvironments.**
At the site of inflammation, ~95% of the myeloid cells are recruited to those sites; thus they need to move against oxygen gradients in order to migrate toward relevant areas of inflammation.
Fig.IV. 10. Schematic representation of the HIF-1α regulated hypoxic response. Stabilization and translocation into nucleus of HIF-1α leading to heterodimer formation with HIF-1β (from: Giaccia, Siim, and Johnson, 2003)
Fig. IV 11. Loss of HIF-1α results in reduced inflammatory responses. The back skin of mice was freed of hair and 5% SDS solution applied epicutaneously once daily for a total of 10 days. Macroscopic appearance of skin after (A) 5 and (B) 10 days of treatment. (C and D) Histological analysis of skin after 5 days of SDS application, H&E, magnification 100. (E and F) Immunolocalization of CD45 in mouse skin after 5 days of 5% SDS treatment. Magnification 200.
Fig. IV. 12. Defective Killing of Group B Streptococcus by HIF-1α macrophages. (A) Engulfment of viable bacteria was characterized by inoculating macrophages with GFP-expressing GBS for 2 hr. Deconvolution fluorescence microscopy was used for documentation. (B) Bone marrow derived macrophages were inoculated with Group B streptococci (GBS) at a MOI of 2.5 and intracellular killing analyzed by determination of viable colony forming units in the macrophage lysates after washing and antibiotic treatment to remove nonengulfed bacteria. ** p<0.01
Fig. 1. Bacterial increase HIF-1α protein expression and stimulate HIF-1 transcriptional activity
A and B, I prepared the bacteria for the assay while CP harvested the murine phagocytes. I designed and executed the bacterial infection studies, harvesting protein, with assistance with CP. CP performed the Western blots using the extracted protein. C, I prepared the bacteria for the assays while CP harvested the murine phagocytes from mice created by A.Deodens. I designed and executed the bacterial infection and CP and I together measured luciferase activity.

Fig. 2. HIF-1α regulates bactericidal activity of myeloid cells.
A through D, I prepared bacteria for the assay while CP harvested the murine phagocytes. I designed and executed all the bacterial infection studies, calculating surviving intracellular CFU, with CPs assistance. E, as above but also E Theodorakis contributed to selection of HIF-1 agonist.

Fig. 3. HIF-1α deletion renders mice more susceptible to GAS infection.
A through D, I took the lead role in design and execution of all in vivo GAS infection studies, including the assessment of the clinical course of infection, quantitative culture, and harvesting tissues for histopathology. CP assisted me directly in these studies.

Fig. 4. HIF-1α is not critical for neutrophils endothelial transcytosis or oxidative burst function.
A and B, Histopathology was performed by CP. C, I took lead in designing and execution of transeptosis assay with assistance with CP. D, CP and I contributed equally to performance of oxidative burst assay with the flow cytometry assistance of N. Hurtado-Ziola.

Fig. 5. Production of granule protease and of murine CRAMP is regulated by HIF-1α.
I did not participate directly in any of these studies.
Fig. 6. HIF-1α and vHL regulate NO production.

A through E, I prepared the bacteria for the assay while CP harvested the murine phagocytes. I designed and executed the bacterial infection studies, harvesting mRNA, with assistance of CP. CP performed the real-time PCR, nitrate-assays and Western blots.
Conclusion and Future Perspectives

A. Introduction

Group A *Streptococcus* (GAS) exclusively infects humans causing a wide range of suppurative and non-suppurative diseases. The most common manifestation of GAS infection is bacterial pharyngitis or “strep throat”. GAS normally colonizes the nasopharynx of 15%–20% of children (Stevens, 1992); rarely GAS pharyngeal infection can extend into deeper tonsillar tissues to cause paratonsillar or retropharyngeal abscesses. GAS has also been associated with a large variety of skin infections of varying severity, including the localized, purulent infection called impetigo (Bisno and Stevens, 1996). Recent decades have seen a resurgence of more severe invasive GAS infections, especially necrotizing fasciitis (NF). Approximately 10,000 cases of invasive GAS disease occur each year in the United States with ~10% mortality (O’Brien et al., 2002). NF is a deep-seated infection of the subcutaneous tissue with progressive destruction of adipose tissue and fascia (Bisno and Stevens, 1996). Clinical manifestations of NF start with diffuse swelling of the infected region and exeruciating localized pain, followed by formation of bullae filled with serosanguineous fluid (Brogan and Nizet, 1997; Schwartz et al., 1995). Rapidly progressing GAS infections in the facial planes are usually associated with bacteremia (Bouvet, 2001). Without proper intervention there can be rapid tissue destruction and even ‘myonecrosis’ of the underlying muscles tissue (Bisno and Stevens, 1996; Brogan and Nizet, 1997; Stevens, 1992). The majority of cases of NF with or without
myonecrosis are complicated by development of organ failure and streptococcal toxic shock syndrome (STSS) (Efstratiou, 2000; Holm et al., 1997; Stevens, 2000, 1989). Tissue biopsies from these patients may reveal subcuticular, dermal and epidermal necrosis, and cellular infiltration at the site of infection with abundance of neutrophils found scattered among the rapidly growing bacteria.

Epidemiologic studies of GAS have linked M protein serotype M1 to be the leading cause of severe infection (Chatellier et al., 2000; Cockerill et al., 1998). One particular serotype MIT1 GAS clone has disseminated globally and persisted as the leading cause of GAS invasive disease (Aziz et al., 2004). GAS possesses a large contingent of virulence factors, enabling the bacteria to colonize, persist, and evade phagocytic clearance en route to GAS invasive disease (Cunningham, 2000).

A hallmark feature in identification of GAS is the presence of a clear β-hemolytic phenotype observed on sheep blood agar media. The toxin responsible for this phenotype is streptolysin S (SLS). In contrast to the other β-hemolysin (SLO) also produced by GAS, the SLS toxin is an oxygen stable molecule, and is non-immunogenic in the course of natural infection. It has been reported that SLS is the most potent bacterial cytotoxin by weight (Koyama, 1963), with a broad spectrum of cytolytic activity, including RBCs from different species, WBCs (Hryniewicz and Tagg, 1977; Ofek et al., 1972), mammalian cell cultures (Taketo and Taketo, 1966) and cellular organelles such as lysosome and mitochondria (Bernheimer and Schwartz, 1964; Keiser et al., 1964). SLS can exists in intracellular, cell-surface bound and
extracellular forms (Calandra and Cole, 1981; Ginsburg and Grossowicz, 1958), and has been never been fully purified. Cell-free preparation of SLS with substantial cytolytic activity requires heavy molecular weight stabilizing molecules like yeast RNA core or albumin (Calandra and Cole, 1981). The cell damaging mechanism of SLS is thought to consist of alterations in the permeability of the cellular membrane system (Bangham et al., 1965). Recently it has been described that the soluble form of SLS binds and insert into the cell membrane, forming transmembrane channels that are both time and temperature sensitive (Carr et al., 2001).

B. Genetic basis of streptolysin S (SLS) production by GAS

SLS deficient transposon mutants were the first step in the identification and characterization of the genetic basis of SLS production. The transposon insertion site in both the strains M1 and M18 were mapped to a promoter motif upstream of a open reading frame (ORF) encoding for a 53 aa protein, due to its association with SLS expression it was named “streptolysin-associated gene A” or sagA (Betschel et al., 1998). Nizet et al. in a subsequent study generated a non-hemolytic Tn917 transposon mutant in a M49 GAS strain. This transposon mutant when sequenced matched to an uncharacterized ORF that was located downstream to the previously described ORF sagA (Nizet et al., 2000). In the experiments of the same publication, SLS biosynthesis was linked to a contiguous 9-gene operon called designated as streptolysin-associated genes (sagA-sagl) (Nizet et al., 2000). Ahead of this operon was a single promoter sequence motif, and two rho-independent terminators were also identified, one situated
between sagA and sagB and the other one present 50 bp downstream of sagI (Nizet et al., 2000).

ORF saga, which encodes a predicted 53 aa protein, possesses several features encountered in the structural genes of bacteriocin-like prepropeptides. SagA includes a Gly-Gly amino acid motif which is conserved in several bacteriocin-like molecules and is known to immediately precede the cleavage site during maturation processing (van Belkum et al., 1997). SagA, if cleaved at this site, would release a 23 aa leader peptide and a 30 aa propeptide C-terminal sequence. The predicted molecular weight (MW) of the 30 aa propeptide is 2.9 kDa, very close to the previously estimated MW of partially purified SLS (Bernheimer, 1967). The 30 aa propeptide sequence is found to be rich in cysteine, threonine, serine and glycine residues. The ribosomally synthesized post translationally modified small peptide molecules known as bacteriocins possess antimicrobial activity and may be produced by bacteria in competition for microenvironmental niches (Bierbaum and Sahl, 1993; Jack et al., 1995b; Tagg et al., 1976). In several bacteriocins, threonine, serine and glycines are the subject of post-translational modifications including the process of dehydration (Jung, 1991). The modified amino acids can then form thioether bonds with adjacent cysteine residues (Li et al., 1996; Nes and Tagg, 1996; Sahl et al., 1995). The presence of unusual amino acid residues in a partially purified SLS preparation (Alouf and Loridan, 1988; Koyama, 1963) which differ from the predicted aa sequence of SagA may indicate SLS to be post-translationally modified and likely to fall into the bacteriocin-like molecule category (Bierbaum and Sahl, 1993; Yorgey et al., 1994). A string of 5
cysteines at the N-terminal end of the SagA propeptide could make SLS sequencing impossible via Edman degradation (Alouf and Geoffroy, 1988). As encountered in other bacteriocins, N-terminal cysteines usually reflect cyclical thioether bridging or the presence of a 2-oxobutyryl group (Meyer et al., 1994).

GenBank homology show SagA propeptide has close similarity to microcin B17 precursor protein MebA, encoded by the structural gene of microcin B17 biosynthetic locus, *mcbA* (Madison et al., 1997). Microcin B17 is an example of a bacterioeoin, expressed by *E. coli*, in which the prepropeptide MebA undergoes post-translational modification at serines and glycines to form cyclic oxazole and thiazole bonds with adjacent cysteines (Li et al., 1996). These modifications of specific aa residues in bacteriocins are accomplished by gene products which catalize the chemical reactions (e.g. dehydration) of the target amino acid residue (Jack et al., 1995b; Liu, 1994). Microcin B17 synthetase enzyme is a collective product of MebB, MebC and MebD, and is responsible for the enzymatic posttranslational modification of the microcin B17 precursor (preMccb) (Madison et al., 1997; Yorgey et al., 1994). All three proteins making the tripeptide complex microcin B17 synthetase are encoded by genes (*mcbB, mcbC* and *mcbD*) present in the microcin genetic locus (Liu, 1994; Yorgey et al., 1994). GAS SagB, with a predicted cytoplasmic localization, has homologies to MebB, a component of microcin B17 synthetase. These findings suggest SagB may serve as a post-translational modifying enzyme in SLS biosynthesis (Nizet et al., 2000).

A bacterial species making bacteriocins must develop mechanisms to protect itself from autotoxicity, often in the form of immunity protein. GAS SagE is a
probable membrane protein with weak homology to PlnP, a putative bacteriocin immunity protein in *Lactobacillus plantarum* (Diep *et al.*, 1996). The export of bacteriocin peptides across bacterial cytoplasmic membrane is facilitated in an ATP-dependent manner by ABC-type transporters (van Belkum *et al.*, 1997).

In some instances, the transporter complex could also cleave the prepropeptide at the Gly-Gly site from its leader peptide to release the mature peptide (van Belkum *et al.*, 1997). Sequence homologies indicate *sagG-sagI* encodes for an ABC-type transporter complex. GAS SagG contains the ATP-binding region, but we do not have additional structural information to suggest SagG could exert any proteolytic activity to itself cleave the leader peptide at the GG site (Nizet *et al.*, 2000; van Belkum *et al.*, 1997). SagC and SagF are predicted to be membrane-bound proteins with no GenBank homologies; similarly SagD lacks predicted homology in GenBank (Nizet *et al.*, 2000).

A rho-independent terminator sequence is present downstream of *sagA* without an additional promoter motif before *sagB*. The operon structure of the 9-gene *sag* locus for SLS production was documented using RT-PCR to demonstrate the presence of a single long polycistronic mRNA (Nizet *et al.*, 2000). The mechanism by which such a leaky terminator results in the differential transcription of mRNA levels is a conserved feature of several bacteriocin like molecules (Skaugen *et al.*, 1997). Northern blot analysis shows an abundance of a *sagA* transcript of 450 bp (Betschel *et al.*, 1998), compared to a minuscule amount of the downstream gene transcription (*sagB-sagI*). GAS appears to make an excess of the putative structural gene product SagA in
prepropeptide form, without expending the biochemical resources required to produce equimolar amounts of each gene product in the biosynthesis and export pathways (Nizet et al., 2000).

C. Mutational analysis of the GAS operon encoding SLS

In our previous published observations, we described the discovery of the 9-gene locus (sagA-sagI) that was necessary and sufficient for SLS production in GAS (Nizet et al., 2000). We also proposed by GenBank homology analyses that SLS is a small-bacteriocin like peptide made by a genetic locus comprising a putative structural gene (sagA), a possible modifying enzyme gene (sagB), putative immunity gene (sagE) and the ABC transporter complex (sagG-sagI) (Nizet et al., 2000). Transposon mutagenesis specified that sag genes were associated with SLS production (Betschel et al., 1998; Nizet et al., 2000), but did not evaluate the requirement of individual gene components in the expression of SLS. In Chapter I of this dissertation, we used allelic exchange mutagenesis followed by single-gene complementation to demonstrate the unique requirement of the genes sagA, sagB, sagC, sagD, sagF and sagG in the production of SLS.

Homologies indicate the sag operon may possess various important components of a genetic machinery for synthesis of a bacteriocin-like small peptide (Nizet et al., 2000). Bacteriocins are ribosomally-synthesized, post-translationally modified small toxins produced by genetic loci consisting of four essential gene components: namely, the structural gene (encoding for a peptide in prepropeptide form), genes encoding for
the modifying enzyme(s), immunity genes and gene products responsible for the export of the mature peptide (Liu, 1994; Nes et al., 1995; Yorgey et al., 1993). SagA is the putative structural gene in the GAS sag operon, encoding for a 53 aa prepropeptide, GenBank homology suggest SagB to be the modifying enzyme and SagG-SagI to be the ABC exporters (Nizet et al., 2000). Elimination of sagA, sagB and one of the candidate transporter components, sagG, from the sag operon produced a nonhemolytic phenotype or SLS-deficient GAS isogenic mutants. Single gene complementation via an expression vector was sufficient to reintroduce SLS production in these mutants (Datta et al., 2005). Similar results were observed in genes with no evident GenBank homologies including sagC, sagD and sagF. Although the functions of these genes are not well-predicted, allelic mutation confirms the requirement of genes sagC, sagD and sagF in SLS production (Datta et al., 2005). Our observations show complete restoration of the β-hemolytic phenotype in single gene complementation of the sagA, sagC, and sagF genes. Genes which encode for predicted cytoplasmic proteins by the PSORT localization algorithm, as in the case of sagB (putative modifying enzyme) and sagD (unknown function), did not show a total complementation in similar experiments. The cytoplasmic expression of a large quantity of the sagB or sagD gene products might introduce toxicity in GAS, or alternatively these products may need to be produced in proper proportion with other downstream gene product for proper functioning. SagG a part of the predicted ABC transporter, and although it is a membrane protein, overexpression might change the stoichiometric configuration of the unified transport protein formed by SagG, SagH and SagI (Datta et al., 2005).
D. The *sag*E gene encodes an immunity function

The predicted 25.4 kDa protein SagE shares weak homology to a candidate immunity protein PnlP of *Lactobacillus plantarum* (Diep et al., 1996). Multiple tries to complement the *sag*E allelic exchange mutant with single gene were unsuccessful. Due to its immunity protein homology, we hypothesized that elimination of the *sag*E gene from GAS could make the mutant bacteria vulnerable to the lethal effects of active SLS. To allow survival of the mutant bacteria, we believe that *sag*E allelic exchange forces a compensatory mutation elsewhere in the operon to abolish the production of SLS (Datta et al., 2005). Consistent with this hypothesis, we found that allelic exchange of *sag*E restoring the SLS expression is only possible in the presence of SagE *in trans* throughout the process of generating the allelic exchange mutant (Datta et al., 2005). The mechanism of immunity function of gene products in bacteriocin-like genetic loci remains to be explored in detail. Mutation in the immunity genes of the nisin operon (*nis*R and *nis*K) increases the susceptibility of the mutants to the activity of the mature nisin bacteriocin (Kuipers et al., 1993). Unlike the *nis*R mutants which were susceptible to exogenous nisin, GAS *sag*E mutants did not show any detrimental effect on the bacterial growth in the presence of external SLS. Most likely the toxic effect of intracellular SLS is responsible for the compensatory mutation in *sag*E mutants (Datta et al., 2005). These experimental data do show *sag*E to have some immunity function, but the actual requirement of the *sag*E gene in expression of SLS remains undetermined.
E. Site-directed mutagenesis of the SagA prepropeptide

SLS could be related to a bacteriocin like small peptide based on its genetic structure (Nizet et al., 2000; Wescombe and Tagg, 2003). One of the requisite features of the genetic structure in a bacteriocin operon is the presence of structural gene (Jack et al., 1995a; Kuipers et al., 1993). The sagA gene has several features of a structural gene and is predicted to encode a 53 aa product, containing a Gly-Gly cleavage site characteristic of non-lantionine bacteriocins (Jack et al., 1995b) as well as lantionines (Ross et al., 1993). SagA also shares a conserved proline residue at the -3 position to the cleavage site with the MebA, the precursor for the E. coli bacteriocin microcin B17 (Madison et al., 1997). Allelic replacement of sagA and single gene complementation of the mutant to the wild-type SLS production, gave us the tools to study the requirement of several amino acid residues in the production of active SLS. The probable cleavage site preceded by the double glycine and proline at -3 was our starting point for site-directed mutagenesis studies. Mutation of proline –3 near the cleavage site abolished SLS production, demonstrating the proline is necessary for the processing of SLS. On the other hand mutation of Gly just preceding the cleavage site allowed partial recognition by the cleaving peptidase, which is compatible with previously reported findings where Gly-Ala processing sequences are found in other bacteriocins (Jack et al., 1994). The accumulated string of cysteines at the N-terminus of the predicted SLS prepropeptide might be required for thioether bond formation with the post-translationally modified amino acid residues (Madison et al., 1997).
Replacement of two of these cysteines (Cys24 and Cys27) with alanine lead to a nonhemolytic phenotype. These results suggest a possible role of cysteines in the formation of thioether bond which are critical in generation of mature SLS. The potential participation of adjacent serine and glycine in the formation of thiaozole or oxazole bonds as commonly encountered in microcin B17 bacteriocin (Madison et al., 1997) has yet to be established in the mature SLS peptide. The presence of a positively-charged amino acid at the very C-terminal end of SLS is a unique feature in certain lantibiotic bacteriocins (van Kraaij et al., 1998). The importance of electrostatic interactions of the C-terminus lantibiotic with the target membrane for membrane permeabilization requires a positively charge residue (van Kraaij et al., 1998). Mutation of tyrosin positively-charged residue at the C-terminus (Lys53) abolished the hemolytic activity of GAS SLS, which may indicate the significance of Lys (positive-charge) in processing or activity of SagA (Datta et al., 2005).

**F. SagA does not exert a global regulation function on other virulence genes in GAS**

Transposon mutants in M1 and M18 GAS strains show decreased ability of these mutants to produce ulceration despite the fact that these mutant only lacked the expression of SLS and had no pleiotrophic effects on sagA deletion on expression of M-protein, cysteine protease SpeB production, expression of SpeA and hyaluronic acid capsule (Betschel et al., 1998). Several later investigators attributed a regulatory function to the sagA gene, even designated it to be the ‘pleiotrophic effects locus’ or pel
(Li et al., 1999). In contrast, a deletion mutant in M6 GAS strain had no transcriptional effect on the \textit{emm} gene encoding for the M-protein, but did show truncation of the N-terminal portion of M-protein (Biswa et al., 2001). Our precise exchange mutation of \textit{sagA} in the M1T1 GAS background was consistent with the earlier finding by Betschel et al. (Betschel et al., 1998), in that we saw no difference in the expression of M-protein, SpeA, cysteine protease or hyaluronic acid capsule. More recent evidence suggests that untranslated RNA of \textit{emm} and \textit{sic} genes could be regulated by the presence of \textit{sagA} (Mangold et al., 2004). In our ongoing collaborative studies with the research group of Bernd Kriekermeyer in Germany, we are further exploring the possibility regulatory role of the \textit{sagA} ORF through a complete transcriptome analysis of wild-type GAS and the isogenic \textit{ΔsagA} mutant strain.

**G. SLS is an important virulence factor in M1 GAS NF in the mouse model**

A GAS M1T1 strain isolated from a patient with NF and streptococcal toxic shock syndrome was well-characterized and found to express several virulence determinants in GAS like SLO, SpeB (cysteine protease), superantigens SpeA, SpeF, SpeG and SmZ, and is thus genetically representative of the globally-disseminated M1T1 GAS clone commonly isolated in invasive GAS disease (Kansal et al., 2000). The allelic exchange of \textit{sagA} in the M1T1 GAS isolate gave us new genetic tools to test the importance of SLS in pathogenesis of GAS invasive disease. Subcutaneous injection of wild-type GAS generated large necrotic ulcers compared to experiments with its isogenic SLS-deficient mutant \textit{ΔsagA}. In certain cases, wild-type GAS
invasive disease extended deeper, involving the underlying muscle tissue causing ‘myonecrosis’ (Datta et al., 2005). Presence of SLS was also important in the persistence of bacterial culture in the skin and the dissemination into blood. Histological evidence of the skin biopsies indicated wild-type GAS induced activation and recruitment of neutrophils to the site of infection, in contrast to the SLS-deficient mutant where there was minimal tissue damage and very little inflammation as evident by markedly reduced number of inflammatory cells at the site of infection (Datta et al., 2005). These results confirm that SLS functions as a virulence factor, contributing to tissue injury and promoting systemic spread.

H. Direct cell injury and phagocytic resistance contribute to the pathogenic effects of SLS in GAS NF

The necrotic tissue damage by wild-type GAS infection may indicate the direct cytolytic effect of SLS on host cellular structures in epidermal and dermal layers of skin and subcutaneous tissue. SLS is regarded as a most potent bacterial toxin by weight, and partially purified SLS is cytotoxic to variety of mammalian cell culture systems (Alouf and Loridan, 1988; Bernheimer, 1967; Ginsburg, 1999; Koyama, 1963). We used human keratinoocyte cell culture models to study the effect of direct cell injury by SLS. Keratinoocytes exposed to wild-type GAS show a dramatic cell death confirmed by trypan blue staining; in comparison, SLS negative mutants show no particular injury to the monolayer. Complementation of the ΔsagA mutant with the single gene sagA not only restores the β-hemolytic phenotype but also brings back the functional effect
of SLS as well (Datta et al., 2005). Thus our in vitro data show a direct tissue injury by SLS to be a likely factor in the necrotic ulceration produced in the mouse model by wild-type GAS.

We further postulated that SLS plays a role in the persistence of the bacterial culture despite an abundance of neutrophils at the site of infection. This paradoxical phenomenon suggests that SLS functions as an antiphagocytic molecule. Partially purified SLS preparations have been shown to possess leukotoxic properties (Ofek et al., 1970, 1972). We demonstrated in our assays that SLS allowed wild-type GAS to survive and grow in heparinized whole blood obtained from healthy donors, in contrast SLS-negative mutants which were cleared much more efficiently. Opsonized wild-type GAS exposed to purified human neutrophils also show increased survival compared to their SLS-negative counterparts (Datta et al., 2005). Our results explaining the antiphagocytic properties of SLS may shed light on the paradox of persistence of GAS bacteria despite abundance of neutrophils at the site of infection. Therefore, a combination of direct cytotoxicity of SLS and neutrophil-mediated inflammatory damage may contribute to the observed necrosis in NF.

I. SLS homologues in pathogenic GGS, GCS and S. porcinus

Several species of streptococci inhabitat and cause infection in animal species other than humans. For example, group B Streptococcus (GBS), aka Streptococcus agalactiae can cause mastitis in cattle. Group G streptococci (GGS), aka Streptococcus
*dysgalactiae*, can also cause mastis in the milking cattle groups. GGS and group C streptococci (GCS) have been known to cause infections characterized by necrotic tissue damage in canines and horses (Williams, 2003). These infectious diseases in animals caused by GGS & GCS share similar pathological feature of human GAS NF.

In contrast to GAS infection, invasive GGS infection in humans is relatively rare and includes pharyngitis, endocarditis, septic arthritis and septic shock. We reported a series of NF in patients with underlying immunosuppressive disease caused by GGS. Histopathology of the skin biopsy identified severe tissue destruction and an abundance of bacteria growing at the site of infection despite a large contingent of neutrophils (Humar *et al.*, 2002). Crossing species from cattles to humans, GGS and GCS causing invasive infection in humans similar to GAS represent new emerging bacterial diseases (Williams, 2003). Human β-hemolytic GGS and GCS share other important virulence factors with GAS including the antiphagocytic surface M and M-like proteins, streptokinase, SLO, C5a peptidase, and possibly a hyaluronic acid capsule (Chhatwal, 2000; Gaviria and Bisno, 2000; Malke, 2000).

We examined two other β-hemolytic streptococcal species in our studies. *S. porcinus* usually is a cause of endocarditis and lymphangitis in captive swine populations (Katsumi, 1998). This bacterial species has also been reported to be an asymptomatic co-colonizer of females genitourinary tract along with GBS (Ferrieri, 2000). Recently *S. porcinus* has been associated with puerperal sepsis or child birth fever and bacteraemia in neonates (Facklam, 1995). *S. iniae* causes
meningoencephalopathies in fish crosses species and cause cellulitis in fish handlers (Fuller et al., 2001).

The series of GGS NF cases and a high phenotypic similarity of GGS with GAS prompted us to hypothesize that the β-hemolysin in GGS could be a similar molecule to SLS of GAS. Our sequencing data confirm the β-hemolysin of GGS and GCS to be SLS produced by nine gene operons homologous to the GAS versions. Plasmid integration in the putative structural gene sagA of GGS abolished the production of SLS, and these nonhemolytic mutants were found to have decreased virulence in an in vivo mouse model of NF (Humar et al., 2002). Genetic techniques used to identify the SLS structural gene homologue sagA in GGS were also employed to identify a similar homologous gene cluster in β-hemolytic S. porcinus. SagA, the putative structural gene in the sag biosynthetic operons is highly conserved among GAS, GGS, GCS, S. porcinus and S. iniae. The respective leader sequences show essentially complete conservation including the proposed cleavage site P-G-G (Fuller et al., 2002; Humar et al., 2002; Nizet, 2002). On other hand, the C-terminal propeptide exhibits some amino acid substitutions, but conserves the initial string of cysteines, several threonines, and the terminal Lys at position 53 (Nizet, 2002). Small differences in the sequence of the propeptide region among the streptococcal species expressing SLS may conceivably be manifest in differences in the hemolytic activity of respective SLS molecules (Humar et al., 2002). The SLS toxin may not be sufficient to produce NF in all streptococcal species producing SLS, but may require the participation other virulence factors like M-like protein which could facilitate adhesion and evasion of phagocytosis (Datta et al.,
2005). GGS lacks cysteine protease SpeB gene, an important GAS virulence factor, which may also help explain the limitation of severe invasive disease to immunosuppressed patient populations (Humar et al., 2002).

J. Neutralization of SLS could be of therapeutic benefit

There is an increasing prevalence of more severe invasive GAS diseases such as NF, often complicated by streptococcal toxic shock syndrome (Bisno and Stevens, 1996; Davies et al., 1996; Stevens, 1996). Severe tissue injury may be associated with poor outcomes, despite an aggressive therapeutic approach (Stevens, 1999). The mainstays for treatment of GAS infections are β-lactam antibiotics such as pencillin and cephalosporins, and most nonsuppurative GAS infection like pharyngitis and superficial skin infection respond readily to these agents. Recently there are growing number of invasive cases which have failed pencillin treatment, and this failure has been attributed to the physiological status of the bacterium. In the case of NF, GAS reaches stationary phase at a rapid pace, decreasing the expression of penicillin-binding-protein (PBP) (Stevens et al., 1993). Many physicians give a combination therapy of penicillin and clindamycin to combat the rapidly growing bacteria, with the caveat that clindamycin could theoretically blocks protein expression, decrease PBP expression, and lower the sensitivity of the bacterium to penicillin (Stevens, 1999).

We have demonstrated the contribution of SLS in GAS virulence via direct cellular toxicity to human epithelial cells, impairment of phagocytic clearance, and the production of NF and bacteremia in the murine model (Datta et al., 2005). Cytolytic
effects of partially purified SLS have been inhibited by treating the cell culture with phospholipids or trypan blue (Ginsburg et al., 1982; Taketo and Taketo, 1966). There is evidence that the phospholipid dipalmotyl phosphatidylcholine (DPPC), a major component of human lung surfactant (Rooney, 1985), can inhibit the cytolytic activity of the GBS-hemolysin/cytolysin on cultured lung epithelial cell monolayer in dose dependent manner (Nizet et al., 1996). We examined the potential effects of neutralizing the cytolytic activity of SLS by DPPC in our in vitro tissue culture model and our in vivo model of GAS NF. In the presence of DPPC, we saw a marked rescue of cultured keratinocytes from the cytolytic effects of SLS. In vivo, SLS expression is correlated with increase bacterial viability in skin ulcers and the ability of GAS to disseminate into deeper tissue and cause bacteraemia (Datta et al., 2005). The histopathological studies of the skin biopsies demonstrate the paradoxical phenomenon of rapid bacterial proliferation in spite of increased neutrophilic recruitment at the site of infection (Datta et al., 2005). The antiphagocytic property of SLS can explain this paradoxical phenomenon, and we also show that neutralization of the cytolytic activity of SLS by DPPC complete reversed this antiphagocytic effect of SLS. DPPC was seen to protect mice infected with M1T1 GAS specifically by neutralizing SLS, thus reducing ulceration and decreased bacterial survival in the NF model. We conclude that DPPC shows some promise in adjunctive therapy of GAS (and GGS) NF, acting to reduce SLS-mediated cytotoxicity and phagocyte resistance.

In summary, I have utilized a molecular genetic approach to study the unique requirement of individual genes of the sag locus in SLS production by the leading
human pathogen GAS. I have discovered that several genes, including the putative structural gene *sagA*, the probable modifying enzyme gene *sagB*, and the exporter component *sagG* are essential for SLS expression. I have also shown that an immunity function against self-toxicity of SLS production is encoded by the *sagE* ORF. Site-directed mutagenesis established some of the amino acid residues in SagA is necessary for the expression of active SLS. Using my isogenic bacteriologic reagents, I have established that SLS plays a major role in the pathogenesis of invasive GAS infection through a combination of direct injury and phagocytic evasion properties. As the cytotoxic and antiphagocytic effects of GAS could be neutralized through an SLS inhibitor (DPPC), the SLS molecule may represent a promising target for novel therapeutic approaches to combat GAS infections.
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