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PATHOGENESIS AND
IMMUNOTHERAPY OF
STREPTOCOCCAL SEPTICEMIA
AND SHOCK

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Cover photo

Immunohistochemical staining of a cell producing IL8 (brown) after being stimulated with heat-killed viridans streptococci (chains).

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*TO MY FAMILY,
MY HUSBAND*

ABSTRACT

Streptococci have been recognized as important causes of severe sepsis. Increased frequencies of streptococcal toxic shock syndrome caused by group A streptococcus (GAS) have been noted worldwide during the last 15 years. More over the incidence of viridans streptococcal and group B streptococcal (GBS) sepsis in immunocompromised individuals has increased during this time period.

The vast majority of outbreaks of invasive GAS infections have been caused by GAS strains of serotype MIT1. In order to determine whether distinct subtypes of these strains were responsible for the more severe manifestations, we examined 35 MIT1 strains obtained from severe or non-severe cases with respect to genetic diversity, superantigen expression, mitogenic and cytokine-inducing capacity. The study showed that highly related MIT1 strains, some indistinguishable, could cause disease of starkly varying severity. This finding underscores the importance of host factors in the pathogenesis of invasive GAS infections.

Superantigen-induced cytokine responses have been shown to be of major importance in the pathogenesis of invasive GAS infections. Here we investigated whether varying clinical manifestations of either viridans streptococcal sepsis in neutropenic patients or serotype V GBS sepsis were related to differences between isolates in their capacity to induce pro-inflammatory responses. Analyses of cytokine responses elicited by culture supernatants as well as heat-killed bacteria revealed a massive induction of pro-inflammatory cytokines. In contrast to GAS, viridans streptococcal or GBS isolates did not induce any T cell proliferation and no production of the T cell cytokines TNF β or IFN γ could be demonstrated, indicating that these strains did not produce any superantigenic activity. Heat-killed viridans streptococcal and GBS isolates induced a cytokine production profile that closely resembled that of the gram-negative endotoxin with an early potent induction of IL1 β , IL8 and TNF α . Culture supernatants prepared from GBS isolates differed from those prepared from GAS and viridans streptococcus since no induction of IL1 β could be demonstrated. Hence, GBS seems to induce a cytokine induction profile, which is distinct from other streptococci. Importantly, no difference in cytokine-inducing capacity could be found between cohorts of isolates from severe and non-severe cases. These findings underscore the importance of both host and bacterial factors, contributing to the inflammatory response and consequently progression of disease.

Intravenous polyspecific immunoglobulin (Ig) G has been suggested to be an efficient adjunctive therapy for invasive GAS diseases mainly due to its ability to neutralize a wide variety of superantigens, and down-regulate pro-inflammatory cytokine responses. We extended these analyses to determine the relative neutralizing activity in polyspecific IgG, IgM, and IgA preparations against GAS superantigens. The study showed that IgM and IgA were potent inhibitors of specific streptococcal superantigens, and the most efficient neutralization of the superantigen SpeA was achieved by an Ig-preparation containing all three isotypes. These findings may have implications for the optimization of immunoglobulin therapy in invasive streptococcal infections.

This thesis emphasizes the importance of host-pathogen interactions in determining the degree of inflammation and therefore the outcome of disease. Although, there are similarities in cytokine responses to GAS, GBS, and viridans streptococcus, they all have unique cytokine induction profiles.

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ORIGINAL PAPERS

This thesis is based on the following papers, which shall be referred to by their Roman numerals:

- I.** S. Chatellier, N. **Ihendyane**, R. G. Kansal, F. Khambaty, H. Basma, A. Norrby-Teglund, D. E. Low, A. McGeer, and M. Kotb. Genetic relatedness and superantigen expression in group A streptococcus serotype M1 isolates from patients with severe and nonsevere invasive disease. *Infect. Immun.* 2000, 68:3523-34.
- II.** N. **Ihendyane**, E. Sparrelid, B. Wretlind, M. Rembeger, J. Andersson, P. Ljungman, O. Ringdén, B. Henriques Normark, U. Allen, D. E. Low, and A. Norrby-Teglund. Viridans streptococcal septicaemia in neutropenic patients: Role of pro-inflammatory cytokines. *Bone Marrow Transplantation*. In press.
- III.** N. **Ihendyane**, D. E. Low, M. Norgren, M. Sellin, E. Persson, and A. Norrby-Teglund. Invasive and non-invasive serotype V group B streptococcal isolates are potent inducers of pro-inflammatory cytokine responses. Manuscript.
- IV.** A. Norrby-Teglund, N. **Ihendyane**, R. Kansal, H. Basma, M. Kotb, J. Andersson, and L. Hammarström. Relative neutralizing activity in polyspecific IgM, IgA, and IgG preparations against Group A streptococcal superantigen. *Clin. Infect. Dis.* 2000, 31:1175-82.

ABBREVIATIONS

| | |
|--------|--|
| ARDS | Acute respiratory distress syndrome |
| CpG | Cytidine-phosphate-guanosine |
| DPPC | Dipalmitoyl phosphatidylcholine |
| GAS | Group A streptococcus |
| GBS | Group B streptococcus |
| GBS-F | Group B streptococcal Factor |
| GRAB | G-related α 2-macroglobulin-binding protein |
| HLA | Human leukocyte antigen |
| HMGB-1 | High mobility group 1 B box protein |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IVIG | Intravenous polyspecific immunoglobulin G |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| MHC | Major histocompatibility complex |
| MIF | Macrophage migration inhibitory factor |
| PepG | Peptidoglycan |
| PFGE | Pulsed-field gel electrophoresis |
| RAPD | Random amplified polymorphic DNA |
| Rib | Resistance to proteases, immunity, group B |
| SIC | Streptococcal inhibitor of complement |
| SL | Streptolysin |
| Spe | Streptococcal pyrogenic exotoxin |
| SSA | Streptococcal superantigen |
| STSS | Streptococcal toxic shock syndrome |
| TcR | T-cell receptor |
| TLR | Toll like receptor |
| TNF | Tumor necrosis factor |

INTRODUCTION

Sepsis and septic shock are serious manifestations commonly associated with high mortality rates. During the last two decades there has been a marked rise in the prevalence of sepsis cases caused by gram-positive bacteria, and at present they account for approximately 50% of all severe sepsis cases (Martin *et al.* 2003). The main pathogens are *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Staphylococcus epidermis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus viridans*. Some of the most severe disease manifestations, i.e toxic shock syndrome and necrotizing fasciitis, are caused by *S. pyogenes*, i.e. group A streptococcus (GAS). Group B streptococci (GBS) and viridans streptococci are important causes of severe sepsis in immunocompromised individuals.

Severe GAS diseases have long been recognized and the history of GAS disease has been characterized by periodic changes in severity of disease (Quinn 1982). The most severe forms of streptococcal disease and their nonsuppurative sequelae were well known long before the discovery of the bacterium itself, as evident by Hippocrates description of epidemic erysipelas in the 5th century BC (Descamps *et al.* 1994).

“Many were attacked by the erysipelas all over the body when the exciting cause was a trivial accident or a very small wound... Many even while undergoing treatment suffered from severe inflammations, and the erysipelas would quickly spread widely in all directions. Flesh, sinews and bones fell away in large quantities. The flux which formed was not like pus but a different sort of putrefaction with a copious and varied flux... The bones were bared and fell away, and there were copious fluxes. Fever was sometimes present and sometimes absent... There were many deaths. The course of the disease was the same to whatever part of the body it spread. Many lost the arm and the entire forearm. If the malady settled in the sides there was rotting either before or behind. In some cases the entire thigh was bared or the shin and the entire foot. But the most dangerous cases of all cases were when the pubes and genital organs were attacked.”

Today this clinical manifestation would be referred to as necrotizing fasciitis.

A marked attenuation in incidence and severity of invasive GAS infections occurred during the later half of the last century, despite unchanged overall prevalence of colonization and infections (Quinn 1982). However, in the mid to late 1980s there was a striking resurgence of highly invasive GAS infections with high mortality in the USA, Canada, Japan, New Zealand, and several European countries (reviewed in (Cunningham 2000; Efstratiou 2000). Similarly, a pronounced increase in incidence and severity of bacteremia caused by GBS (Jackson *et al.* 1995; Schlievert *et al.* 1993) and viridans streptococci was noted during the same period (Cohen *et al.* 1983; Henslee *et al.* 1984; Catto *et al.* 1987; Kern *et al.* 1987; Menichetti *et al.* 1987; Sotiropoulos *et al.* 1989; Classen *et al.* 1990; Weisman *et al.* 1990 Villablanca, 1990 #523; Elting *et al.* 1992; Bochud *et al.* 1994; Persson *et al.* 2000).

Classification of Streptococci

Streptococci are gram-positive cocci arranged in chains of varying length. Classification of streptococci is based on their hemolysis pattern on blood agar plates following 24 hours incubation, and there are three distinct types comprising alpha (incomplete greenish hemolysis), beta (complete hemolysis) and gamma (no hemolysis) (Figure 1) (Brown 1919). The streptococci can be further subdivided into Lancefield's serogroups, A-H and K-V, based on the composition of their cell wall carbohydrate antigens (Figure 1) (Lancefield 1933).

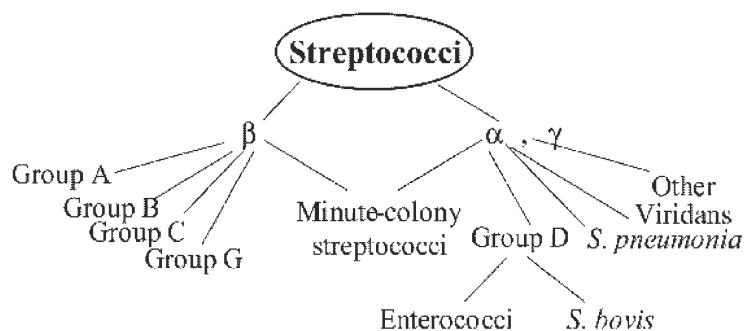


Figure 1. Classification of streptococci.

Lancefield group A β -hemolytic streptococcus, *S. pyogenes*, is the most important human pathogen in this group. GAS are subtyped into specific serotypes based on the identification of the cell-surface associated M and T proteins (Lancefield 1962), and to date more than 100 different serotypes have been verified by *emm*-gene nucleotide sequencing (Facklam *et al.* 1999). Certain serotypes have been suggested to be associated with specific disease manifestations, such as the M1 and M3 strains that are commonly associated with severe invasive GAS infections.

S. agalactiae is the species designation for streptococci belonging to Lancefield group B, and most strains show a narrow zone of β -hemolysis but some are non-hemolytic. The GBS organism was identified in 1935 by Lancefield and Hare in vaginal cultures from asymptomatic post-partum women (Lancefield *et al.* 1935). Definitive identification of GBS is based on detection of the group B-specific cell wall antigen common to all strains. The polysaccharide “S” substance of the cell capsule has allowed additional classification into distinct serotypes and nine serotypes have so far been described: Ia, Ib, II-VIII. The GBS serotypes I, II and III have been recognized as the most frequent cause of invasive disease in neonates (Berner 2002), and the serotype V has recently emerged as a cause of invasive disease and seems to be especially prevalent among non-pregnant adults (Figure 2) (Blumberg *et al.* 1996).

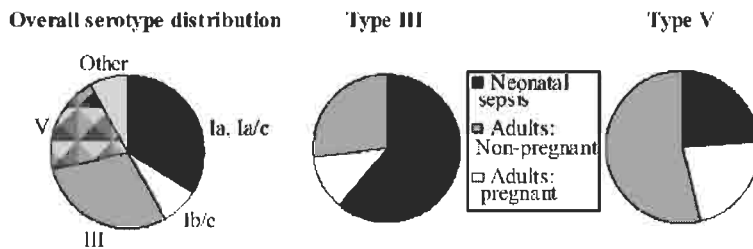


Figure 2. Distribution of group B streptococcal serotypes in Atlanta, 1992-93. The figure is based on the data presented by Blumberg *et al.* (Blumberg *et al.* 1996).

The viridans streptococcal group is a heterogeneous collection of α -hemolytic and nonhemolytic streptococci, which includes *S. mutans*, *S. sanguis*, *S. salivarius*, *S. intermedius*, and *S. mitis*. Their group name is derived from *viridis* (Latin for “green”) since many of these bacteria produce incomplete lysis seen as a green pigment on blood agar media. In contrast to the β -hemolytic streptococci, serological test are of limited use in the identification of viridans streptococcal subspecies since there is a high degree of cross-reaction and lack of definite antigens.

GROUP A STREPTOCOCCAL INFECTIONS

Throat, skin, and systemic infections

GAS is a major causes of bacterial infections, giving rise to a wide spectrum of diseases of which pharyngotonsillitis and impetigo are the most common (Bisno 1991). Colonization of the upper respiratory tract with GAS often present as pharyngotonsillitis, and less often as sinusitis or otitis media. It is estimated that between 2-6% of the population are asymptomatic carriers of GAS (Begovac *et al.* 1993; Christenson *et al.* 1997). Suppurative complications may occur and include bacteremia, meningitis, pneumonia, peritonsillar abscesses, endocarditis, and peritonitis (Stevens 1992; Weiss *et al.* 1997). Scarlet fever, which is usually associated with pharyngeal infection, is characterized by fever, rash, desquamation, and in the severe cases also marked systemic toxicity (Bisno 1991).

Puerperal sepsis, also known as “childbed fever”, is mainly caused by GAS in post-partum mothers (McGregor *et al.* 1984; Ooe *et al.* 1997). This used to be a very severe life-threatening disease, which was responsible for up to two thirds of deaths of post-partum mothers in the late 18th and 19th centuries (Gergis *et al.* 1999). Classical puerperal sepsis occurs within 24 to 48 hours after the delivery, but GAS puerperal sepsis may also occur during the pregnancy that involves a big risk for both mother and child (Ooe *et al.* 1997). However, the incidence of puerperal sepsis has decreased during recent years.

Skin infections caused by GAS include impetigo (pyoderma), a confined superficial infection of the skin that primarily affects exposed areas (i.e. face, arms, legs). Erysipelas and cellulitis are skin tissue infections that are manifested by local signs of inflammation (pain, erythema, and warmth) and, in most cases, by fever and leukocytosis. Erysipelas involves the superficial layers of the skin and cutaneous lymphatics and the erythema is well demarcated from surrounding skin, whereas

cellulitis extends more deeply into the subcutaneous tissue and the demarcation between involved and non-involved skin is not distinct. Necrotizing fasciitis is the most severe tissue infection caused by GAS, and affects deep subcutaneous tissue and fascia. It is characterized by an extensive progressive destruction of fascia and fat, and is often associated with marked systemic symptoms (Bisno *et al.* 1996). More severe skin and tissue infections may be complicated by septicemia.

The most severe invasive GAS infection is streptococcal toxic shock syndrome (STSS). STSS was defined by “The Working Group on Severe Streptococcal Infections” (Working Group on Severe Streptococcal Infections 1993). Patients are considered to have STSS if they have GAS bacteremia, and hypotension in combination with two or more of the following: acute renal failure, coagulation abnormalities, liver abnormalities, acute respiratory distress syndrome (ARDS), generalized rash and soft tissue necrosis. Some STSS patients also experience soft tissue inflammation and pain, as well as non-specific symptoms such as fever, chills, malaise, nausea, vomiting, and diarrhea (Stevens 1992; Davies *et al.* 1996). Although persons of all age groups are susceptible to STSS, increased age has been recognized as an underlying factor (Stevens 1992; Davies *et al.* 1996). Other underlying conditions found to be associated with STSS include human immunodeficiency virus infection, cancer, renal failure, leukemia, heart or pulmonary disease, alcohol and intravenous drug abuse (Torres-Martinez *et al.* 1992; Stevens *et al.* 1996; Weiss *et al.* 1997; Eriksson *et al.* 1998). Despite the fact that most patients receive prompt anti-microbial therapy, the mortality rates of STSS remain high, ranging between 30 and 80% (Stevens 1992; Stevens 1995; Davies *et al.* 1996).

Post-streptococcal complications

Patients may also develop immune-mediated post-streptococcal sequelae, such as erythema nodosum, reactive arthritis, rheumatic fever, and acute glomerulonephritis. Rheumatic fever is a non-suppurative complication with onset several weeks after throat infections caused by GAS. It is characterized by inflammatory changes involving the heart, joints, blood vessels, and subcutaneous tissues (Krisher *et al.* 1985; Bisno 1991). Acute glomerulonephritis is also a non-suppurative complication of GAS disease, which is usually seen one to three weeks after GAS-associated skin or throat infection. The disease is characterized by acute inflammation of renal glomerulus with edema, hypertension, hematuria, and proteinuria. There are data suggesting that

nephritogenic and rheumatogenic GAS serotypes exist, however, this association is not completely exclusive and there is an overlap between serotype and disease. Not all strains of a given M serotype are equally nephritogenic or rheumatogenic (Bisno 1991).

Group B streptococcal infections

GBS was originally known for causing bovine mastitis (Brown 1920; Stableforth 1938) and was first reported as a human pathogen in 1935 (Fry 1938). The GBS organism is part of the commensal flora of the gastrointestinal and genital tracts, and approximately 15-35% of women are asymptotically colonized with GBS (Schuchat *et al.* 1990; Berner 2002). The bacteria may cause sepsis in newborns by passage from the colonized mothers to their infants during labor and delivery (Schuchat 1998).

GBS infection in neonates

Neonatal sepsis is divided into early onset and late onset syndrome, the former being more frequent and associated with higher mortality rates ranging between 4% and 15% as compared to 2 and 6% for late onset infections. The early onset infection is defined as the development of systemic infection during the first six days of life. In these cases, the newborns have acquired GBS intrapartum from their mothers either before or during birth. The major clinical manifestations of early onset GBS disease are bacteremia, pneumonia, respiratory distress and meningitis (Schuchat 1998). The late onset infections usually have an onset of disease from seven days to three months of age. Meningitis and bacteremia without focus are the most common clinical manifestations.

GBS infection in adults

In recent years, invasive GBS infections have also been recognized as a major problem in pregnant and non-pregnant adults, especially in those who have underlying diseases (Farley *et al.* 1993; Jackson *et al.* 1995; Domingo *et al.* 1997). The most common disease manifestations include skin and soft tissue infections, septicemia, urinary tract infections and osteomyelitis. Gardam *et al.* (Gardam *et al.* 1998) reported three cases of necrotizing fasciitis in adults, and one of the cases also fulfilled the criteria for STSS, they all had underlying disease such as diabetes and leukemia. Risk factors associated with invasive GBS disease in non-pregnant adults include among others diabetes, cancer, stroke, renal failure, and human

immunodeficiency virus infection (Farley *et al.* 1993; Domingo *et al.* 1997; Schuchat 1999). The incidence of GBS in non-pregnant adults increases with age and is highest in patients over 60 years of age.

Viridans streptococcal infections

Viridans streptococci are the predominant species of the human oral flora and commonly inhabit the upper respiratory, gastrointestinal, and female genital tracts. These bacteria may also be found in the skin flora. Viridans streptococci are considered to be of low virulence, rarely causing severe disease in immunocompetent hosts. However, they may occasionally cause bacteremia and subacute endocarditis, and some subgroups, in particular *S. intermedius*, even invasive pyogenic infections (Johnson *et al.* 2000).

Viridans streptococci have become recognized as an increasingly important cause of bacteremia in neutropenic patients undergoing chemotherapy (Bochud *et al.* 1994; Gonzalez-Barca *et al.* 1996; Marron *et al.* 2000; Razonable *et al.* 2002). Occasionally these patients develop alpha-streptococcal shock syndrome, which is characterized by hypotension, ARDS, multi-organ failure, and high mortality rates (Cohen *et al.* 1983; Henslee *et al.* 1984; Catto *et al.* 1987; Kern *et al.* 1987; Menichetti *et al.* 1987; Sotiropoulos *et al.* 1989; Classen *et al.* 1990; Weisman *et al.* 1990; Villablanca *et al.* 1990; Elting *et al.* 1992; Bochud *et al.* 1994; Razonable *et al.* 2002). Alpha-streptococcal shock syndrome seems to be exclusive for the immunocompromised host since it has never been reported in immunocompetent individuals.

PATHOPHYSIOLOGY OF SEPSIS

Severe sepsis develops as a consequence of microbial antigenemia inducing a generalized activation of numerous host defense systems, including adaptive and innate immune responses of which the complement, coagulation, contact-phase, and fibrinolytic systems are prominent contributors (Figure 3) (Bone 1991; Esmon *et al.* 1999). Bacterial components that activate these systems include, among others, the gram-negative lipopolysaccharide (LPS), gram-positive cell wall components, as well as secreted toxins and enzymes (Srisikandan *et al.* 1999). Activation of the pro-inflammatory and pro-coagulatory cascades results in the release of pro-inflammatory cytokines, endothelins, tissue damaging proteinases, lipid mediators (platelet activating factor, arachidonic acid, and eicosanoids), and hypotensive molecules such as kinins

and nitric oxide (Bone 1991; Esmon *et al.* 1999). Many of these mediators regulate cellular and humoral immune responses and are essential for an adequate and efficient host defense against infecting microorganisms. However, excessive and dysregulated release of these mediators is the key event leading to the clinical symptoms seen in sepsis and shock, such as circulatory collapse, organ failure and death. The initial pro-inflammatory phase of sepsis is followed by a second phase characterised by an anti-inflammatory state, commonly referred to as the compensatory anti-inflammatory response syndrome, which is believed to be a mechanism by which the host tries to minimize tissue damage caused by dysregulated inflammation (Vincent 2000). This may be achieved by release of anti-inflammatory cytokines, as well as by down-regulation and shedding of cytokine receptors. However, the exact actions of this anti-inflammatory system in the pathophysiology of sepsis have not yet been clearly established.

Cytokines and sepsis

The cytokines most commonly associated with sepsis are interleukin (IL) 1, tumor necrosis factor α (TNF α), IL6, IL8, IL12, interferon γ (IFN γ), macrophage migration inhibitory factor (MIF), and high mobility group 1 B box (HMGB-1) protein (Bernhagen *et al.* 1998; Wang *et al.* 1999; Andersson *et al.* 2000; Cavaillon *et al.* 2000). However, there are several more cytokines released during sepsis, which in addition to those mentioned above, interact in a complex network involving several interaction points and feedback loops. The cytokine cascade is initiated by IL1 and TNF α , secreted from activated macrophages in response to pro-inflammatory microbial components, and these cytokines are therefore often referred to as “early mediators of sepsis” (Figure 3). Activation of the cytokine cascade results in a rapid activation of different cell types, further release of IL1 and TNF α and several other important mediators (Cavaillon *et al.* 2000).

IL1 and TNF α induce potent pyrogenic and hypotensive responses, and administration of either cytokine in animal models reproduces the clinical symptoms of sepsis (Cavaillon *et al.* 2000). IL8 is a highly powerful chemokine, which attracts and activates polymorphonuclear leukocytes, and has been suggested to have a central role in the pulmonary inflammation resulting in ARDS (Cavaillon *et al.* 2000).

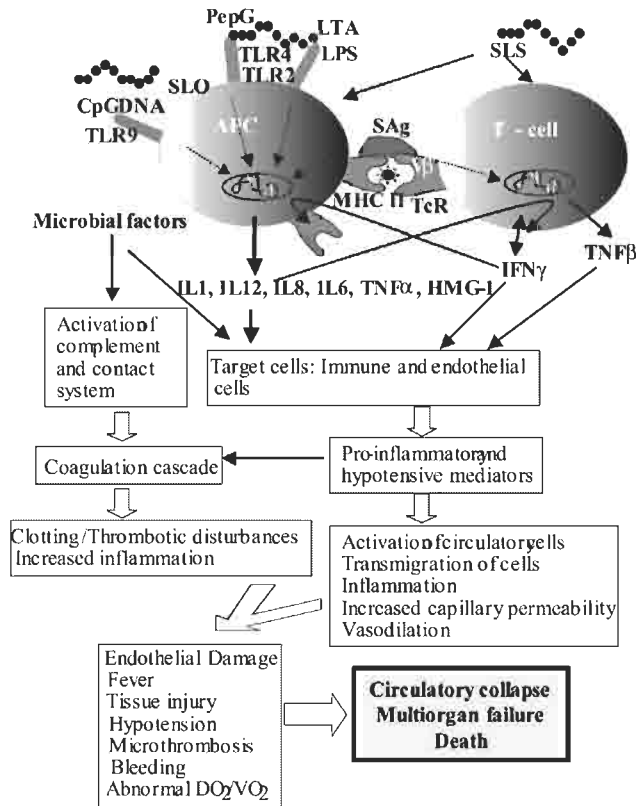


Figure 3. Pathophysiology of bacterial sepsis. The figure schematically outlines the interaction between host cells and some of the major microbial factors, including lipopolysaccharide (LPS) produced by gram-negative bacteria, and peptidoglycan (PepG), lipoteichoic acid (LTA), streptolysins O and S (SLO and SLS), and superantigens (SAg) produced by gram-positive bacteria, as well as cytidine-phosphate-guanosine (CpG) DNA. The microbial factors interact with specific host receptors, such as major histocompatibility complex class II molecules (MHC II) and Toll-like receptors (TLR) on antigen presenting cells (APC) and T cell receptor (TcR) on T cells. This triggers activation of the cells and release of the pro-inflammatory cytokines, which subsequently results in activation of the cytokine cascade and the coagulation system. Activation of these host defense system leads to excessive and dysregulated release of inflammatory mediators and consequently the clinical symptoms of sepsis.

MIF is a pituitary- and macrophage-derived factor (Bernhagen *et al.* 1998) that acts as a pro-inflammatory cytokine, and was recently shown to be a critical mediator of septic shock (Calandra *et al.* 2000). HMGB-1 is a nuclear DNA-binding protein, which was recently demonstrated to be a cytokine since it stimulates pro-inflammatory responses

in monocytes/macrophages, is produced during inflammatory responses *in vivo*, and is required for full expression of inflammation in endotoxemia models (Wang *et al.* 1999; Andersson *et al.* 2000). Wang *et al.* (Wang *et al.* 1999) identified HMGB-1 as a late mediator of endotoxic shock in mice. HMGB-1 was found at increased levels in serum during a pro-longed period following LPS-administration and antibodies to HMGB-1 protected against LPS-induced lethality, whereas administration of HMGB-1 to mice was lethal (Wang *et al.* 1999).

PATHOGENESIS OF STREPTOCOCCAL INFECTIONS

Virulence factors of GAS

The ability of GAS to cause infection is dependent upon a large number of virulence factors, including a variety of membrane bound structural molecules as well as secreted toxins and enzymes. These factors interact with immune cells and other factors of the host to enhance virulence of the bacteria (Figure 3).

Cell surface proteins of GAS

Adherence and colonization are prerequisites for infection to occur, and GAS express several virulence factors that promote these events, including adhesins, capsule, C5a peptidase, and the M and M-like proteins (Cunningham 2000). Adherence of the bacteria can be mediated by several cell surface associated molecules such as lipoteichoic acid (LTA), M protein, protein F, Sfb1 protein, fibronectin-binding protein (FBP54), serum opacity factor, hyaluronic acid capsule, glyceraldehyde-3-phosphate dehydrogenase, vitronectin binding protein, a 70kDa galactose-binding protein and collagen-binding proteins (Cunningham 2000).

Among the most important surface proteins, is the M protein, which exhibits potent anti-phagocytic activity (Cunningham 2000). This activity has been suggested to be partly mediated through binding of the M protein to the plasma protein factor H or factor H-like protein 1, which are regulatory proteins of the alternative complement pathway that can inhibit the deposition of C3-derived opsonins (Horstmann *et al.* 1988; Johnsson *et al.* 1998). However, a recent report by Kotarsky *et al.* (Kotarsky *et al.* 2001) showed that GAS phagocytosis resistance was equally high in streptococci that lacked the major binding sites for factor H and factor H-like 1 protein. Hence, additional mechanisms are likely contributing to the anti-phagocytic activity of the

M-protein, among others interaction with the C4b-binding protein (Berggard *et al.* 2001).

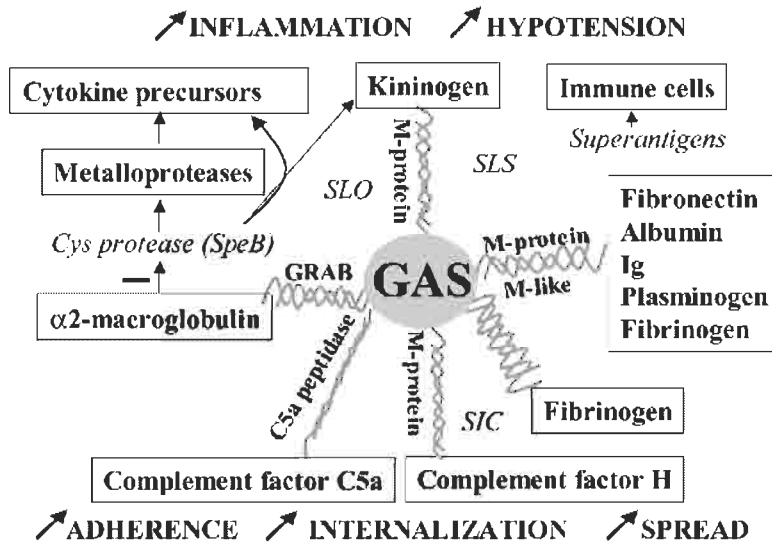


Figure 4. Virulence factors of group A streptococcus (GAS). GAS produce both cell-surface associated molecules (indicated in bold) and extracellular factors (indicated in italic) that interact with human proteins and immune cells (boxed). This leads to reduced phagocytosis, increased bacterial adherence, internalization, spread of the bacteria and consequently host inflammation and hypotension. Cys protease *SpeB*, streptococcal cysteine protease; Ig, immunoglobulin; GRAB, G-related α 2-macroglobulin-binding protein; SLO, streptolysin O; SLS, streptolysin S; SIC, streptococcal inhibitor of complement.

Opsonic antibodies directed against the N-terminal region of M-proteins confer protection against infection, but the protection is type-specific and the individual remains susceptible to infection by other serotypes (Lancefield 1962). GAS also express M-like proteins that are structurally related to the M proteins (Heath *et al.* 1987; Lindahl 1989; Gomi *et al.* 1990; Åkesson *et al.* 1990; Bessen *et al.* 1992; Frick *et al.* 1994; Åkesson *et al.* 1994; Boyle 1995; Cunningham 2000). Many M-like proteins have immunoglobulin-binding properties and can interact with the Fc portion of IgG and/or IgA (Cunningham 2000). Several of these proteins also exhibit specificity for other human proteins such as albumin, kininogen, and plasminogen (Ben Nasr *et al.* 1995). Rasmussen *et al.* (Rasmussen *et al.* 1999) identified a novel surface protein, which was named G-related α 2-macroglobulin-binding protein

(GRAB) based on its capacity to bind α_2 -macroglobulin. Since α_2 -macroglobulin is the most abundant protease inhibitor present in serum, the authors proposed that this could generate a local protection of the bacteria from degradation by proteases produced by phagocytic cells or by the streptococci itself.

Like other gram-positive bacteria, a major constituent of the GAS cell wall is peptidoglycan (PepG). It is composed of alternating *N*-acetyl glycosamine and *N*-acetyl muramic acid sugar chains that are interlinked by peptide bridges resulting in a large complex macromolecular structure (Schleifer *et al.* 1972). During bacterial infection, PepG and several other cell wall components are thought to be involved in the inflammatory reaction (Kengatharan *et al.* 1998). PepG has been shown to activate complement (Greenblatt *et al.* 1978; Mattsson *et al.* 1994), granulocytes (Guthrie *et al.* 1984; Martinez-Martinez *et al.* 1993) and also to up-regulate expression of adhesion molecules on endothelial cells (Martinez-Martinez *et al.* 1993). It also induces production of proinflammatory cytokines, such as IL1, IL6 and TNF α , by monocytes *in vitro* (Timmerman *et al.* 1993; Heumann *et al.* 1994; Mattsson *et al.* 1994; Majcherczyk *et al.* 1999). The stem peptides of PepG induce a 100-fold higher pro-inflammatory response than intact PepG (Majcherczyk *et al.* 1999).

LTA consists of sugar phosphate polymers that extend through the cell wall onto the surface of the gram-positive cell and have been suggested to mediate bacterial adherence to the host's epithelial cells. Both PepG and LTA have been shown to activate leukocytes and trigger production of pro-inflammatory cytokines and chemokines, as well as other inflammatory mediators such as inducible nitric oxide synthetase (Bhakdi *et al.* 1991; Mattsson *et al.* 1993; Timmerman *et al.* 1993; Heumann *et al.* 1994; Danforth *et al.* 1995; Cleveland *et al.* 1996; Morath *et al.* 2001). It has been demonstrated that PepG, LTA and lipoproteins from gram-positive bacteria activate innate immunity through Toll-like receptors (TLR), in particular TLR2 (Medzhitov *et al.* 2000; Beutler *et al.* 2001; Imler *et al.* 2001; Kimbrell *et al.* 2001). Recognition of these TLR2 ligands have been shown to be facilitated by CD14 (Weidemann *et al.* 1994; Cleveland *et al.* 1996; Gupta *et al.* 1996) and a role for LPS binding protein was recently described in the recognition of pneumococcal PepG (Weber *et al.* 2003). The specificity of the TLR sensing results from the co-operation between TLRs; TLR2 and TLR6 are both recruited to the macrophage phagosome

where they recognize PepG, whereas TLR1/TLR2 respond to bacterial lipoproteins (Ozinsky *et al.* 2000; Takeuchi *et al.* 2002). Engagement of the TLRs triggers intracellular signal transduction pathways, resulting in activation of the transcription factor NF- κ B and subsequent induction of expression of proinflammatory genes (Medzhitov *et al.* 2000; Beutler *et al.* 2001; Kimbrell *et al.* 2001). Thus, gram-positive cell wall components activate innate immunity by the same signalling pathway as the cell wall component LPS does, the principal mediator of gram-negative sepsis, which interacts with TLR4 (Medzhitov *et al.* 2000; Beutler *et al.* 2001; Kimbrell *et al.* 2001).

Extracellular virulence factors of GAS

Superantigens have been recognized as pivotal mediators of the systemic symptoms seen in severe invasive GAS disease. GAS produce several exotoxins with superantigenic activity including streptococcal pyrogenic exotoxins (Spe) A-C, F-J, M-L (Kotb 1992; Kotb 1995; Proft *et al.* 1999; Proft *et al.* 2001; Smoot *et al.* 2002), streptococcal superantigen (SSA) (Mollick *et al.* 1993; Reda *et al.* 1996), streptococcal mitogenic exotoxin (Sme) Z, SmeZ2, and SmeZ3 (Kamezawa *et al.* 1997; Proft *et al.* 1999; Gerlach *et al.* 2000; Proft *et al.* 2000). The superantigens bind, without prior cellular processing, to the V β domain of the T cell receptor (TcR) and to major histocompatibility complex (MHC; human leukocyte antigen (HLA)) class II molecules outside the antigen binding cleft (Figure 3) (Marrack *et al.* 1990; Kotb 1995). Superantigens bind specifically to a set of T cells expressing certain V β -chains. Although there is overlap in V β -specificities among different superantigen, each superantigen has specificity for a unique V β repertoire. Interaction with the V β -region makes it possible for superantigens to cross-link T cells with MHC class II molecules on antigen presenting cells even when the TcR does not recognize the bound antigenic peptide (Marrack *et al.* 1990). Thus, superantigens by-pass the normal rules for antigen presentation and T cell recognition, and can therefore activate up to 20 % of the resting T cell population compared with 0.0001-0.01% for a conventional antigen. Superantigen cross-linking of the T cell receptor and MHC molecules on antigen presenting cells results in activation of both cell-types and subsequent massive production of pro-inflammatory cytokines, such as IL1, TNF α , TNF β , and IFN γ (Kotb 1995). Since cytokines have been shown to be mediators of sepsis and septic shock, the central role for superantigens in the pathogenesis of severe invasive manifestations is

thought to be attributed mainly to their ability to induce excessive production of pro-inflammatory cytokines.

Studies on clinical materials have provided evidence for the involvement of superantigens and their induction of cytokines in the pathogenesis of severe invasive GAS infections (Watanabe-Ohnishi *et al.* 1995; Norrby-Teglund *et al.* 2001). Determination of T cell receptor V β -repertoires in STSS patients demonstrated V β -specific alterations of the T cell repertoire during the acute phase, thus, providing evidence for an *in vivo* effect of superantigens (Watanabe-Ohnishi *et al.* 1995). Furthermore, superantigens have been identified in the plasma of STSS patients (Sriskandan *et al.* 1996), as well as at the local site of infection in patients with deep tissue infections (Norrby-Teglund *et al.* 2001). Analyses of cytokine responses in circulating cells and tissue biopsies from the acute phase of infection revealed a direct correlation between magnitude of cytokine responses and the severity of infection (Norrby-Teglund *et al.* 2000; Norrby-Teglund *et al.* 2001).

GAS express two cysteine proteases, the classical cysteine protease SpeB and the newly discovered IdeS. Both these proteolytic enzymes have been shown to degrade human immunoglobulins, and consequently inhibit immunoglobulin-mediated opsonophagocytosis (Collin *et al.* 2001; Collin *et al.* 2002; Eriksson *et al.* 2003). In addition, numerous other physiologically important human proteins are substrates for SpeB including IL1 β -precursor (Kapur *et al.* 1993), metalloproteases (Burns *et al.* 1996), the extracellular matrix proteins vitronectine and fibronectin (Kapur *et al.* 1993), and kininogens (Herwald *et al.* 1996). SpeB can also degrade and cleave streptococcal surface proteins, among others the M-protein and the C5a peptidase (Berge *et al.* 1995; Raeder *et al.* 1998). The regulation of proteolysis seems to be tightly regulated at the bacterial surface, through expression of the protein GRAB, which as mentioned earlier binds to α 2-macroglobulin (Figure 4). Rasmussen and Björck recently proposed that the initial phase of a GAS infection is characterized by inhibition of proteolysis and complement activity at the bacterial surface, whereas at a later stage proteolytic activity will facilitate bacterial spread by release of bacterial surface proteins and degradation of human tissues (Rasmussen *et al.* 2002).

Another mechanism by which IdeS interferes with phagocytosis is through its homology with the α -subunit of Mac-1, a human leukocyte β 2 integrin that is important for the innate immunity to microbial infection (Lei *et al.* 2001). IdeS is therefore also

referred to as the GAS Mac 1-like protein and was shown to bind to CD16 (FcγRIIIB) on human neutrophils with a subsequent inhibition of opsonophagocytosis and bacterial killing.

The streptococcal inhibitor of complement (SIC) protein is yet another important secreted factor that is synthesized predominantly by M1 GAS strains (Åkesson *et al.* 1996). As the name alludes to, SIC interferes with the complement system by inhibiting the formation of the membrane attack complex (Åkesson *et al.* 1996; Fernie-King *et al.* 2001). Interestingly, the *sic* gene showed a unique degree of polymorphism (Stockbauer *et al.* 1998; Hoe *et al.* 1999), and this hypervariability was found to be generated by natural selection on mucosal surfaces (Hoe *et al.* 1999). A recent report by Frick *et al.* (Frick *et al.* 2003) demonstrated that SIC, aside from its complement inhibition, was a potent inhibitor of the two human antimicrobial peptides involved in bacterial clearance, neutrophil α-defensin and LL37. Importantly, the SIC from M1 strains, commonly associated with outbreaks of severe invasive GAS infections, was more potent in this respect as compared to SIC from M12 and M55 strains that are rarely involved in major outbreaks.

GAS produces two hemolysins, streptolysin S and O (SLS and SLO) that are responsible for the hemolysis on blood agar plates. SLS is an oxygen-stable, non-immunogenic, cell-bound hemolysin that can lyse erythrocytes, leukocytes, and platelets. It has been demonstrated that tissue damage associated with the formation of necrotic skin lesions in mice depends on the activity of SLS (Betschel *et al.* 1998). SLO is a secreted protein produced by nearly all clinical GAS isolates tested (Alouf 1980; Muller-Alouf *et al.* 1997; Shiseki *et al.* 1999). SLO interacts with cholesterol in target cells to form multi-subunit pores and is cytolytic for several cell types, including erythrocytes, leukocytes, macrophages, and platelets (Hirsch *et al.* 1963; Launay *et al.* 1979). It has been shown that SLO acts in synergy with SpeA, which results in increased production of the pro-inflammatory cytokines IL1β and TNFα (Hackett *et al.* 1992). SLO also acts synergistically with the cysteine protease SpeB, as evident by increased lung injury in a rat model (Shanley *et al.* 1996).

In addition to the above virulence factors, there are also immunostimulatory unmethylated oligonucleotides containing cytidine-phosphate-guanosine (CpG) motifs (Chatellier *et al.* 2000; Wagner *et al.* 2000). Unmethylated CpG DNA is exclusively found in prokaryotes, and has been shown to activate macrophages,

dendritic cells, and B-cells, thereby triggering production of IL1, IL6, TNF α , IL12, IL10, and Th1 type of cytokines (Krieg *et al.* 1995; Klinman *et al.* 1996). Activation of cellular responses by CpG DNA was recently shown to be mediated by TLR9 (Hemmi *et al.* 2000).

Virulence factors of GBS

The type specific polysaccharide capsule of GBS is an important virulence factor by virtue of its anti-phagocytic properties, and isogenic capsule-negative mutants demonstrated reduced virulence in a murine model (Edwards *et al.* 1980; Rubens *et al.* 1987). Wessels *et al.* (Edwards *et al.* 1982; Wessels *et al.* 1989) showed a critical role for the capsular sialic acid in the inhibition of the alternative complement pathway-mediated opsonophagocytosis (Edwards *et al.* 1982; Wessels *et al.* 1989). Type-specific antibodies to the capsular polysaccharide overcome this inhibitory effect with subsequent efficient bacterial clearance (Edwards *et al.* 1979; Rubens *et al.* 1987; Wessels *et al.* 1989). The basis for capsule mediated inhibition of opsonophagocytosis is not fully defined, but it has been suggested to involve prevention of C3b deposition and conversion of C3b to the inactive form iC3b (Marques *et al.* 1992) as well as inhibition of C5a production (Takahashi *et al.* 1999).

The C-protein is a cell surface protein that is composed of two immunologically distinct antigens, the α and β proteins (Bevanger *et al.* 1979), and which is commonly expressed on the surface of GBS serotypes Ia, Ib, and II, but not on type III (Johnson *et al.* 1984; Stålhammar-Carlemalm *et al.* 1993). Antibodies to the C-protein protect against lethal infection with C-protein expressing GBS strains (Lancefield *et al.* 1975), and inactivation of the α C-protein resulted in attenuated virulence of the bacteria (Li *et al.* 1997). A recent study by Bolduc *et al.* (Bolduc *et al.* 2002) suggested that the α C-protein has an important role in mediating adherence to and internalisation into epithelial cells of GBS.

Another important protective surface protein is the protein Rib (resistance to proteases, immunity, group B), which is distinct from but related to the α C-protein (Stålhammar-Carlemalm *et al.* 1993). The Rib protein is expressed by type III, V and VIII GBS strains (Stålhammar-Carlemalm *et al.* 1999; Lachenauer *et al.* 2000). GBS also express several α -like (Alp2) and Rib-like (R28/Alp3) proteins that elicit protective immunity. The expression of these proteins varies in different capsular

serotypes (Areschoug *et al.* 1999; Stålhammar-Carlemalm *et al.* 1999; Lachenauer *et al.* 2000). The exact function of these proteins remains to be defined, but R28 of GAS, which is also a member of this family of proteins, has been shown to promote interaction with human epithelial cells (Stålhammar-Carlemalm *et al.* 1999).

The β C-protein is also of interest since it interacts with both IgA and factor H, which suggests that it can influence the host's innate immune responses (Russell-Jones *et al.* 1984; Lindahl *et al.* 1990; Areschoug *et al.* 2002).

GBS also produce extracellular virulence factors, including the C5a peptidase, β -hemolysin, and GBS-F (Hill *et al.* 1988; Bohnsack *et al.* 1991; Puliti *et al.* 2000; Ring *et al.* 2000; Henneke *et al.* 2001; Talati *et al.* 2001; Doran *et al.* 2002; Henneke *et al.* 2002). The C5a peptidase is highly conserved among streptococci and was first described for GAS (Wexler *et al.* 1985). It inactivates the human chemotaxin, C5a, and thereby reduces the chemotactic response to infection. The GBS β -hemolysin is a pore-forming cytotoxin capable of injuring a broad range of eukaryotic cell types (reviewed in (Nizet 2002)). A correlation between the level of GBS β -hemolysin expression and the GBS injury to human lung epithelial cells has been demonstrated (Nizet *et al.* 1996). Other potentially important activity of GBS β -hemolysin shown *in vitro* include induction of nitric oxide production and apoptosis in macrophages (Ring *et al.* 2000; Henneke *et al.* 2002), activation of human brain endothelial cells resulting in production of IL8 and other activation markers, and invasion and activation of lung epithelial cells (Doran *et al.* 2002). Animal models have confirmed an important role for the β -hemolysin in the pathogenesis of GBS disease (Nizet *et al.* 1997; Puliti *et al.* 2000; Ring *et al.* 2002). Another pro-inflammatory molecule secreted by GBS was recently described by Henneke *et al.* (Henneke *et al.* 2001), who found a heat-labile secreted factor named GBS-F, which induced potent inflammatory responses in murine macrophages through interaction with TLR2, TLR6 and CD14.

Virulence factors of viridans streptococcus

Viridans streptococci are usually organisms of low virulence and typically cause life-threatening infection only when the oral mucosa is disrupted and the host's defense mechanisms are compromised, as in neutropenic cancer patients. Viridans streptococci bind to oral mucosa, tooth surfaces, and dental plaque via interaction between specific microbial and host receptors (van Houte 1983). LTA from viridans

streptococci has been suggested to be an important adhesin through interaction with fibronectin (Lawrance *et al.* 1990). Little is known about the mechanisms involved in the pathogenesis of viridans streptococcal septic shock and ARDS in immunocompromised patients. A study by Engel *et al.* (Engel *et al.* 1996) showed increased IL6 levels in sera of patients with alpha-streptococcal shock syndrome as compared to uncomplicated bacteremia cases. Both cell wall preparations and cell-free bacterial supernatants derived from viridans streptococci have been shown to induce production of pro-inflammatory cytokines, such as IL1 β , IL8, and TNF α in human cells (Bhakdi *et al.* 1991; Heumann *et al.* 1994; Soto *et al.* 1996; Hanage *et al.* 2002).

HUMORAL IMMUNITY AGAINST GAS VIRULENCE FACTORS

Analyses of the outbreaks of GAS infections in the late 1980s, which were caused predominantly by MIT1 strains, revealed that invasive cases, regardless of severity, had significantly lower antibody levels against the M1-protein than did healthy controls or pharyngotonsillitis cases (Holm *et al.* 1992; Basma *et al.* 1999). Since severe and non-severe invasive cases had equally low levels of opsonic M1-antibodies, the data suggested that low humoral immunity to the M1-protein rendered the individuals susceptible to invasive infection by MIT1 strains, but did not influence severity of systemic manifestation.

Antibody levels against GAS superantigens were also assessed in patient materials collected during these outbreaks (Holm *et al.* 1992; Norrby-Teglund *et al.* 1994; Basma *et al.* 1999; Eriksson *et al.* 1999; Mascini *et al.* 2000). A Swedish study reported lower levels of neutralizing antibodies against SpeB and SpeF in patients with severe invasive GAS disease as compared to pharyngotonsillitis cases, whereas no difference in neutralization of SpeA was noted (Norrby-Teglund *et al.* 1994). Similarly, two other studies reported higher anti-SpeB reactivity in plasma from controls than in severe invasive cases (Eriksson *et al.* 1999; Mascini *et al.* 2000). However, these two latter studies also reported a relation between more severe cases and lower titers of anti-SpeA antibodies. These somewhat conflicting results may be due to several factors, including strain variation in superantigen expression and the use of different assays measuring either amount of the specific antibodies or their biological function. The lack of correlation between ELISA antibody titers against

GAS superantigens and the biological function, i.e. neutralizing activity of the antibodies, has previously been demonstrated (Norrby-Teglund *et al.* 1994). Basma *et al.* (Basma *et al.* 1999) analyzed a clinical GAS material for neutralizing antibodies against specific toxins and against culture supernatants prepared from each patient's own isolate. The study showed that although there was no significant difference in neutralizing activity against specific superantigens between controls and patients with invasive infection, the patients had significantly lower immunity towards their own isolate as compared to the controls. Equally low neutralizing activity was detected in plasma from severe or non-severe cases, indicating that although low immunity to the patient's own isolate contributes to susceptibility to the superantigens, it does not explain the varying clinical manifestations. Collectively the studies demonstrate a crucial role for antibodies against GAS virulence factors in protection against invasive infection.

IMMUNOGLOBULIN THERAPY OF SEVERE INVASIVE GAS INFECTIONS

The high mortality rates of severe invasive GAS infections demonstrate a definite need for adjunctive therapy in these diseases. The correlation between low protective humoral immunity against several important GAS virulence factors and invasive infection indicates that immunoglobulin therapy might be a potential therapeutic strategy. However, in order for such a therapy to be efficacious, a broad specificity would be required to cover the various GAS serotypes as well as the whole repertoire of superantigens and other virulence factors produced by GAS. Commercial human intravenous immunoglobulin (IVIG) preparations exhibit a very broad polyspecificity since they are generated from pools of sera from more than a thousand blood donors (Mouthon *et al.* 1996; Ballow 1997).

It has been reported that IVIG preparations contain opsonic antibodies to a variety of bacterial strains (Yang *et al.* 1989; Fisher *et al.* 1994; Hiemstra *et al.* 1994; Weisman *et al.* 1994), including GAS (Basma *et al.* 1998) and GBS (Fischer *et al.* 1992; Weisman *et al.* 1994). Furthermore, *in vitro* experiments have demonstrated that IVIG contains antibodies against a broad spectrum of staphylococcal and streptococcal superantigens (Takei *et al.* 1993; Skansen-Saphir *et al.* 1994; Norrby-Teglund *et al.* 1996; Norrby-Teglund *et al.* 1996; Darville *et al.* 1997; Norrby-Teglund *et al.* 1998). These antibodies were found to completely abolish the

proliferative and cytokine inducing capacity of the superantigens (Takei *et al.* 1993; Skansen-Saphir *et al.* 1994; Norrby-Teglund *et al.* 1996). Inhibition of superantigen-induced cytokine production was seen even when IVIG was added 24 hours after initiation of superantigen stimulation, which implied that there are additional mechanisms, beside direct antigen neutralization, that contribute to the reduced cytokine production (Andersson *et al.* 1994). A general immunosuppressive effect has been described for IVIG, and is thought to be mediated partly by Fc-interaction, upregulation of anti-inflammatory cytokines, and presence of soluble immune components, such as sCD4, sCD8, and human leukocyte antigens (HLA) class II molecules (Mouthon *et al.* 1996; Ballow 1997) (figure 5). A recent study by Samuelsson *et al.* (Samuelsson *et al.* 2001) identified the Fc inhibitory receptor for IgG, Fc γ RIIB, as an important mediator of the therapeutic benefits of IVIG. In a murine model of immune thrombocytopenia, they showed that IVIG-treatment lead to an increase in the number of macrophages expressing Fc γ RIIB, and subsequent inhibition of platelet destruction and protection against disease (Samuelsson *et al.* 2001).

IVIG therapy has been a useful treatment modality since its introduction in the early 1980s. It has many clinical indications, including several autoimmune and immunodeficiency diseases (Mouthon *et al.* 1996; Ballow 1997), and holds promise in many additional medical conditions, including severe invasive GAS infections (Mouthon *et al.* 1996; Norrby-Teglund *et al.* 1998).

Several case reports have shown clinical improvement of patients with streptococcal and staphylococcal toxic shock treated with IVIG (Barry *et al.* 1992; Nadal *et al.* 1993; Lamothe *et al.* 1995; Mahieu *et al.* 1995; Ogawa *et al.* 1995; Chiu *et al.* 1997; Perez *et al.* 1997). Kaul *et al.* (Kaul *et al.* 1999) performed an observational cohort study of IVIG-therapy in STSS patients, and found a significant reduction in case fatality rate in patients treated with IVIG as compared to non-treated controls. Furthermore, analyses of superantigen neutralizing activity in patients' plasma pre- and post-therapy revealed that the majority of patients significantly increased their plasma neutralizing activity post-therapy. In addition, a significantly reduced TNF α and IL6 production was found in post-therapy blood as compared to pre-samples (Kaul *et al.* 1999).

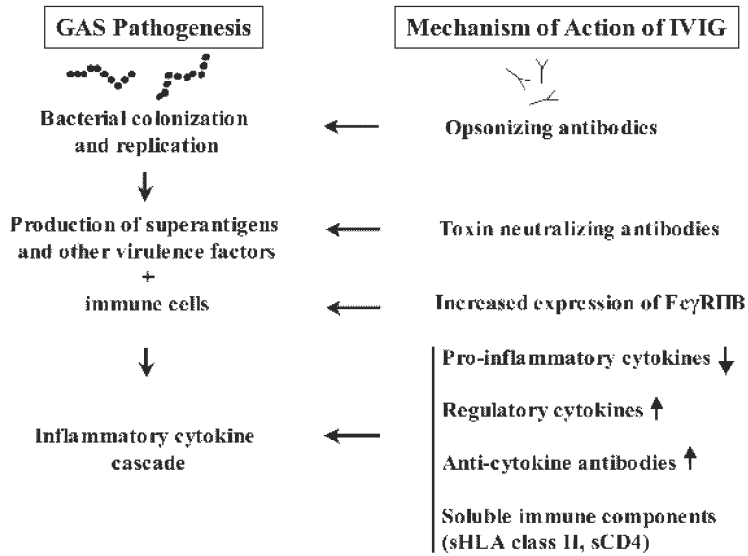


Figure 5. Mechanisms of action of intravenous immunoglobulin (IVIG) in group A streptococcal (GAS) infection.

Further support for the use of IVIG in STSS was provided by a European multicenter placebo-controlled trial (Darenberg *et al.* 2003). Due to a low incidence of STSS during the study period, patient recruitment was slow, and the trial was prematurely terminated after enrolment of 21 patients. The results revealed a trend towards a reduced mortality rate in IVIG-treated cases as compared to those receiving placebo (10% versus 36%) (Darenberg *et al.* 2003). Importantly, this trend in reduced mortality was supported by significantly better improvement of organ dysfunction, following treatment days 2 and 3 in the IVIG group, whereas no such improvement could be seen in the placebo group.

AIMS OF THE STUDY

The overall work of this thesis was to:

- Determine whether specific subclones of group A streptococcal strains are responsible for the more severe invasive diseases.
- Compare the cytokine induction profiles of GAS, GBS, and viridans streptococcal strains in order to identify common pathogenic mechanisms.
- Conduct *in vitro* studies of the action of IVIG therapy in invasive GAS infections.

MATERIAL AND METHODS

BACTERIAL STRAINS

Paper I: This paper is based on a clinical material collected during 1994 – 1996 through active surveillance of invasive GAS infections in Ontario, Canada. GAS strains of MIT1 serotype isolated from patients with severe invasive infection (n=21), i.e. STSS and/or necrotizing fasciitis, or non-severe invasive GAS infection (n=14), i.e. no hypotension or multiorgan failure, were analyzed.

Paper II: A retrospective screening of stem cell transplanted and leukaemic patients during 1995-2002 was performed in order to identify neutropenic patients with severe and non-severe sepsis. Nine neutropenic patients who had severe sepsis syndrome with or without ARDS were identified and their strains included in the study. These isolates were compared with 10 viridans streptococcal isolates from non-severe septic episodes in neutropenic patients. The material included two cases, who each experienced two septic episodes, one severe and one non-severe, caused by different viridans streptococcal strains.

Paper III: The study included five GBS isolates, which had caused neonatal sepsis (blood isolates) and four invasive (blood or tissue cultures) isolates from non-pregnant adults with necrotizing fasciitis. All four adult patients had underlying conditions, including diabetes mellitus and leukemia. Four isolates from vaginal swabs of colonized asymptomatic women were also included in the study. Three of the invasive strains from adults were collected in Toronto, whereas the 10 remaining isolates were all collected in Sweden. The GBS isolates were all of serotype V.

Paper IV: One GAS strain of serotype T3/B3264 isolated from a blood culture of an STSS patient at Huddinge University Hospital Stockholm was used in this study.

PREPARATION OF BACTERIAL CULTURE SUPERNATANTS AND HEAT-KILLED BACTERIA

The isolates were cultured overnight to stationary phase in Todd-Hewitt broth and bacterial growth was determined by plating of serial dilutions of the culture on blood agar plates. Bacteria from stationary phase cultures were harvested by centrifugation,

and proteins in cell-free supernatants were ethanol precipitated, resuspended in H₂O, dialyzed, and filter sterilized.

The bacterial pellet was resuspended in PBS, heat-killed by boiling for 10 minutes, and washed repeatedly.

CHARACTERIZATION OF GAS STRAINS

Molecular characterization

Genomic DNA was analyzed by random amplified polymorphic DNA (RAPD) analysis, and pulsed-field gel electrophoresis (PFGE) using two different enzymes *SmaI* and *SfiI*, respectively. Strains were considered identical if their banding patterns were identical, and highly related if the patterns differed by one band.

The *emm1*, *sic*, and *speA* genes were amplified by PCR and nucleotide sequenced.

The presence of genes encoding the superantigens SpeA, SpeB, SpeC, SpeF, SpeG, SpeH, SmeZ, and SSA was detected by PCR amplification using primer pairs specific for each gene.

In vitro superantigen expression

Proteins present in culture supernatants of GAS isolates were detected by western blot analysis using antibodies specific for SpeA, SpeB, and SpeF and quantification of the Spe amount was achieved by scanning of the autoradiograms on a PhosphorImager and subsequent densitometrical analyses.

IG PREPARATIONS

Five commercially available IVIG preparations, Gammagard S/D[®], IVIG-CP[®], Pentaglobin[®], IgAbulin[™], and IgA colostrum with varying content of IgG, IgM, and IgA were tested (Table 1). In addition, purified IgM and IgA preparations were also included in the study. The Ig-preparations were analyzed for the presence of anti-Spe antibodies of IgG, IgM, or IgA isotype using ELISA.

Table 1. Composition of IgM, IgA and IgG preparations

| Immunoglobulin preparation | IgG % | IgM % | IgA % |
|------------------------------|-------|-------|-------|
| Pentaglobin | 76 | 12 | 12 |
| IgM (Hu299) | 5 | 82 | 13 |
| IgAbulin | 25 | 0 | 75 |
| IgA (purified from IgAbulin) | <0.1 | <0.1 | 99.9 |
| IgA colostrums 1 | NA | NA | 96 |
| IgA colostrums 2 | NA | NA | ≥98 |
| IVIG-CP | 97 | 2.5 | <0.05 |
| Gammagard S/D | >99 | - | <0.01 |

CELL CULTURE EXPERIMENTS*Cell preparation and culture*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors by Ficoll-Hypaque gradient centrifugation. PBMC (1×10^6 cells/ml) were stimulated with purified superantigens, sterile culture supernatants, or heat killed bacteria for various lengths of time. In the phospholipid inhibition experiments (paper III), 0.5mg/mL of phospholipid dipalmitoyl phosphatidylcholine (DPPC) (Sigma-Aldrich, Sweden) was prepared by sonication and added to the cell cultures as previously described (Nizet *et al.* 1996).

Proliferation and neutralization assay

Proliferative responses were assessed after 72 hours of culture by ³H-thymidine incorporation. Phytohemagglutinin-L (PHA) was used as a positive control. Neutralization of toxins by IVIG was determined by relating the proliferative response in cultures containing various concentrations of Ig to the response obtained in the presence of fetal bovine serum.

Cytokine analyses

Simultaneously with the proliferation assay, the cell cultures were tested for cytokine production using an intracellular immunostaining technique (Andersson *et al.* 1992). Cells were harvested at various time points, transferred to adhesion glass slides, and fixed with 2% formaldehyde. Cytokine production was assessed by intracellular

immunohistochemical staining using anti-cytokine specific antibodies. Intracellular staining was achieved by addition of the permeabilizing agent saponin. Biotinylated secondary antibodies were applied followed by an avidin-peroxidase complex (Vectastain Elite, Vector Lab) and the 3'-diaminobenzidine tetrahydrochloride (DAB) substrate. All cytokine producing cells, except the IL1 family, show a characteristic juxtannuclear staining pattern, representing accumulation of the cytokine to the golgi organelle, whereas IL1-producing cells show a cytoplasmic staining pattern (Andersson *et al.* 1992).

The concentrations of IL1 β , IL8, and TNF α in cell culture supernatants were determined by Luminex cytokine multiplex analyses using the Fluorokine MAP kits (R&D, Minneapolis, MN) or the Bio-Plex cytokine assay kits (Bio-Rad Laboratories, Inc.) and the Luminex¹⁰⁰ instrument (Luminex corp., Austin, Tx).

Serum cytokine assay

IL8 and IL1 β levels in sera obtained during the septic episode from neutropenic patients were measured by automated chemoluminescence immunoassay (IMMULITE[®], DPC, CA, USA).

STATISTICAL ANALYSES OF DATA

Student *t* test, Mann-Whitney *U* test, Kruskal Wallis, or Wilcoxon matched pairs test analysis were used to evaluate significant differences of the data. *P* < 0.05 was considered significant.

RESULTS AND DISCUSSION

HIGHLY RELATED GAS STRAINS CAUSE INVASIVE DISEASE OF STARKLY VARYING SEVERITY

Since the late 1980s a dramatic increase in incidence and severity of invasive GAS infections has been reported in the USA, Canada, Japan, New Zealand, and several European countries (reviewed in (Cunningham 2000; Efstratiou 2000)). This dramatic resurgence of highly aggressive invasive GAS infections might either be due to changes in virulence properties of the bacteria and/or alterations of host protective immunity against specific strains or specific virulence factors. Epidemiological data demonstrated that the vast majority of these outbreaks were caused by GAS strains of the same serotype, i.e. M1T1 (reviewed in (Efstratiou 2000)). Ongoing active surveillance in Ontario, Canada, revealed a large number of severe, i.e. STSS and/or necrotizing fasciitis, and non-severe cases caused by M1T1 strains. In this study (I), we investigated whether these strains causing invasive disease of starkly varying severity were similar or whether distinct subtypes were responsible for the more severe diseases. We examined the genetic diversity, superantigen expression, and mitogenic- and cytokine-inducing capacity of 35 M1T1 GAS strains, 21 severe, i.e. STSS and/or necrotizing fasciitis, and 14 non-severe cases. The data showed that all 35 M1T1 GAS isolates were highly related as they had identical *emm1.0* gene sequences, the *speA* 2 allele, and the same *spe* genotype. In addition, the isolates had the same RAPD profile, and most had identical PFGE banding pattern after digestion with two enzymes.

A high degree of *sic* gene variation was found among the M1 isolates, but there was no link between any specific mutation, deletion or insertion and the severity of disease. These results are in complete agreement with previous reports, which demonstrated that the *sic* gene is hypervariable (Perea Mejia *et al.* 1997; Stockbauer *et al.* 1998; Hoe *et al.* 1999). Variations in *Spe* expression were also found among the isolates, but again no particular pattern was significantly associated with the more severe disease manifestations.

Furthermore, comparison of the mitogenic and cytokine-inducing activity of the isolates, revealed that all strains were equally potent inducers of proliferative and cytokine responses. In agreement with previous studies (Norrby-Teglund *et al.* 1994; Norrby-Teglund *et al.* 1997), PBMCs from different individuals responded differently

to the GAS supernatants in both proliferation and cytokine production assays. However, despite this inter-individual variation, PBMCs from each individual responded similarly to isolates from severe and non-severe cases.

Thus, the study showed that highly genetically related, sometimes indistinguishable, GAS strains could cause disease of varying severity in different individuals, and highlighted the importance of host factors in determining the outcome of infection. Basma *et al.* (Basma *et al.* 1999) analyzed acute phase sera from these patients infected with genetically related MIT1 strains, and found that the invasive cases had significantly lower protective antibody levels against superantigens and against the M1-protein, as compared to healthy age-matched geographical controls. Importantly, patients with invasive disease, severe or non-severe, had equally low levels of protective antibodies in acute phase sera. These data suggested that although protective immunity contributed to susceptibility to invasive disease, it did not determine the severity of invasive infection (Basma *et al.* 1999). It was later shown that the severity of infection was strongly associated with the propensity of the individual to respond with a high or a low inflammatory response upon challenge with streptococcal superantigens (Norrby-Teglund *et al.* 2000). This was demonstrated by Kotb *et al.* (Kotb *et al.* 2002) to be a consequence of the HLA class II type of the patients as certain HLA class II haplotypes conferred strong protection against severe systemic GAS disease, whereas others were associated with risk of severe disease. Furthermore, they demonstrated a relation between HLA class II type and level superantigen-induced inflammatory response, with the risk haplotypes promoting significantly higher cytokine responses as compared to the protective haplotypes.

CLINICAL GBS AND VIRIDANS STREPTOCOCCAL ISOLATES ARE POTENT INDUCERS OF PRO-INFLAMMATORY RESPONSES

GBS and viridans streptococcus have become recognized as increasingly important causes of sepsis in immunocompromised individuals (Schlievert *et al.* 1993; Bochud *et al.* 1994; Jackson *et al.* 1995; Gonzalez-Barca *et al.* 1996; Marron *et al.* 2000). To examine whether varying clinical manifestations of GBS or viridans streptococcus sepsis could be related to differences in pro-inflammatory activity among isolates, we identified cohorts of patients who had experienced varying severity of infection caused by these bacteria.

Viridans streptococcal sepsis (II)

Viridans streptococci frequently cause sepsis in neutropenic patients, and in 3 –33% of the cases, the infection develops into alpha-streptococcal shock, characterized by shock and ARDS. We chose to compare strains from neutropenic patients with severe viridans streptococcal sepsis and patients who experienced only bacteremia and fever but no organ involvement. Retrospective screening identified nine neutropenic patients with severe sepsis, including seven cases with ARDS, i.e. alpha-streptococcal shock. These isolates were compared to 10 isolates from 10 non-severe septic episodes in neutropenic patients. A major strength of the patient material, although small in numbers, was that it included two patients who had experienced two septic episodes, one severe and one non-severe, caused by different viridans streptococcal strains.

The immunostimulatory activity of heat-killed bacteria as well as cell-free bacterial culture supernatant containing extracellular proteins, such as exotoxins, hemolysins and CpG DNA, was determined by *in vitro* cell culture using human PBMC. The induction of IL1 β -, TNF α -, and IL8-production was studied due to their well established association with severe sepsis and septic shock, and IFN γ - and TNF β -production were included as markers for superantigen-triggered T-cell activation.

The data showed that both bacterial supernatants and heat-killed bacteria from all studied strains were potent inducers of the pro-inflammatory cytokines IL1 β and IL8. However, heat-killed bacteria were significantly more potent inducers of cytokine production than the cell-free bacterial supernatants. In contrast to superantigen containing supernatants from GAS, viridans streptococci did not induce any T cell proliferation and no production of the T cell cytokines TNF β or IFN γ could be demonstrated after stimulation with either heat-killed bacteria or culture supernatants. This lack of T cell activation implied that the tested viridans streptococcal strains did not secrete any superantigens, which is in agreement with a previous study that also reported lack of induction of T cell proliferation by viridans streptococcus (Soto *et al.* 1998). However, in a study by Matsushita *et al* (Matsushita *et al.* 1995) superantigenic activities by an extracellular product of *S. mitis* was reported. Hence, we cannot rule out the possibility that certain viridans streptococcal strains may produce superantigens.

Surprisingly, none of the isolates induced TNF α -production after 24 hours of stimulation with either supernatant or heat killed-bacteria. Since the cytokine induction profile of LPS is characterized by an early production of TNF α with peak production at 4-6 hours (Andersson *et al.* 1992), we wanted to examine whether viridans streptococci followed similar kinetics. Indeed, a high production of TNF α was found in cultures stimulated with heat-killed bacteria for 4h. Thus, heat-killed viridans streptococci have a similar cytokine induction profile as LPS with high expression of early TNF α production, high expression of IL1 β and IL8 after 24 hours stimulation, and lack of, or only very low, induction of T cell derived cytokines. It seems likely that the pro-inflammatory activity induced by heat-killed bacteria may be mediated by PepG and LTA, since they have previously been shown to induce pro-inflammatory cytokine production through the Toll-like receptor signalling pathway (Heumann *et al.* 1994; Standiford *et al.* 1994; English *et al.* 1996). However, future studies are required to define the major pro-inflammatory molecules and their host receptors.

Culture supernatants were also potent inducers of IL1 β and IL8, and most (90%) induced detectable but low levels of TNF α . Future studies should attempt to identify the factors responsible for this pro-inflammatory activity, and potential candidates include shedded cell wall components, exotoxins, such as hemolysins, and/or CpG DNA. The study shows that surface-associated molecules and secreted factors of viridans streptococci are powerful inducers of pro-inflammatory cytokines, and may be important for development of severe sepsis.

Importantly, we could not demonstrate a difference in cytokine inducing capacity between the strains causing severe and non-severe sepsis. However, as mentioned above, the material contained two patients who each experienced two episodes of sepsis, one severe and one non-severe. During the first episode the patients developed ARDS and during the second episode they only reacted with fever and elevated CRP, but had no signs of organ involvement. The patients remained neutropenic during both episodes, and comparison of these matched isolates showed that the isolates from the severe sepsis episodes consistently induced higher frequencies of IL1 β - and IL8-producing cells as compared to the isolate from the non-severe episode. These differences between severe and non-severe isolates did not result from bacterial growth variations, since viable counts of the cultures showed equal numbers from severe or non-severe isolates.

Another difference between the severe and non-severe patient cohorts, was demonstrated in sera from 3 severe cases as compared to 5 non-severe cases, where the IL8-levels were higher in the severe cases. These *in vivo* data indicate that the higher IL8-production seen after *in vitro* stimulation may be clinically relevant. Although few bacterial strains and few patient sera were available for analyses, collectively the data indicate that in addition to host factors, there may be differences in pathogen properties that could partly explain the varying disease manifestations and specifically the development of ARDS. The study emphasizes the need for studies using a customized approach in which the patient's response to its own isolate is evaluated.

Group B Streptococcal Sepsis (III)

In recent years an important change in the serotype distribution of GBS causing invasive disease has occurred with the emergence of serotype V being reported from the United States, France, and Sweden (Blumberg *et al.* 1996; Harrison *et al.* 1998; Lin *et al.* 1998; Areschoug *et al.* 1999; Le Thomas-Bories *et al.* 2001; Smith *et al.* 2002). GBS of serotype V has been recovered from sepsis patients, including neonates, pregnant and non-pregnant adults, and seems to be especially prevalent among non-pregnant adults (Blumberg *et al.* 1996). It was therefore of interest to focus on this newly emerging serotype, and determine the cytokine induction profile of clinical type V isolates.

The results showed that heat-killed bacteria prepared from all GBS isolates induced high levels of IL1 β and IL8, but no TNF α , after 24 hours of stimulation. TNF α -production was found after 4 hours of stimulation, and immunostaining revealed that the TNF α response induced by whole bacteria was predominantly attributed to CD68-positive cells and not to T cells. Overall, the cytokine induction profile of heat-killed GBS was highly similar to that of LPS, suggesting that the pro-inflammatory activity is mediated by cell wall components such as lipoproteins and peptidoglycans that signals through the TLR receptors. Recent reports have shown that, in contrast to other gram-positive cell wall components, GBS cell wall is not recognized by TLR2 (Flo *et al.* 2000; Henneke *et al.* 2001; Henneke *et al.* 2002). It has been further shown that GBS cell wall products induce pro-inflammatory cytokines via activated MyD88-dependent signal transduction pathway (Henneke *et al.* 2001). This implies that GBS cell wall components are recognized by other TLRs, but not by TLR1, 2, 4, 6, and 9 as has been shown using macrophages from respective knockout mice (Henneke *et al.*

2001; Henneke *et al.* 2002). Hence, the receptor for GBS cell wall products remains as of yet unknown.

Surprisingly, none of the GBS culture supernatants induced IL1 β -production. This finding was verified by immunohistochemical staining at the single cell level, which revealed only background levels of IL1 β -producing cells (0 – 1%) following stimulation with cell-free GBS supernatants, whereas 7-8-fold higher frequencies were observed with heat-killed GBS. In contrast, high production of IL8 was induced by GBS culture supernatants, indicating that there was no overall inhibition of responses of the supernatant.

In an attempt to identify the pro-inflammatory molecule(s) in the supernatant, we assessed the influence of β -hemolysin by addition of DPPC, which has been shown to be a potent inhibitor of the GBS β -hemolysin inhibitor (Tapsall *et al.* 1991; Nizet *et al.* 1996), to the cell cultures. However, the IL8 response was unaffected by the addition of DPPC, and hence β -hemolysin did not seem to be the main mediator of the IL8 response. Recent studies have identified a heat-labile immunologically active factor (GBS-F), which requires the expression of TLR2 and 6, TLR adapter molecule, MyD88, and CD14 (Henneke *et al.* 2001; Henneke *et al.* 2002). Hence, GBS-F was another likely candidate, and we therefore tested the effect of heat-inactivation of the supernatant. The results showed an almost complete abolishment of the IL8-response, which confirmed that GBS-F was present in the supernatant and contributes to the pro-inflammatory response.

Similar to our previous findings with GAS and viridans streptococci, we could not demonstrate any significant difference with respect to pro-inflammatory activity between isolates of the three patient cohorts, as they all induced equally potent cytokine production. This finding together with the noted inter-individual variation, which has also been reported in response to GAS and viridans streptococcal factors (papers I-II (Norrby-Teglund *et al.* 1994; Norrby-Teglund *et al.* 1997)), indicate that host-pathogen interplay strongly affects the magnitude of cytokine responses and thereby the outcome of disease manifestation.

Unique cytokine induction profiles of GAS, GBS and viridans streptococcus

Comparison of the cytokine induction of GAS, GBS, and viridans streptococcal strains revealed unique cytokine induction profiles for each subtype (Figure 6). Superantigenic activity was only found among the GAS strains, as evident by

profound T cell activation. The heat-killed streptococcal preparations showed a high degree of similarities with high TNF α already after 4 hours, and high IL1 β and IL8 after 24 hours. Induction of cytokines by culture supernatants varied significantly among the three streptococcal subtypes. The most dramatic variation was the lack of IL1 β -induction by GBS supernatants, which was evident both by intracellular immunostaining of IL1-producing cells and by analyses of secreted cytokines in the culture supernatant. The reason for this lack of IL1 β response is as of yet unknown, but will be addressed in future studies. Importantly, the results of papers I-III show that cell-wall components and secreted factors of GAS, GBS and viridans streptococci are potent inducers of pro-inflammatory cytokines, and hence most likely contribute to the pathogenesis of sepsis caused by these bacteria. Identification of variations in cytokine induction profiles may be important with respect to the efficacy of immunotherapy.

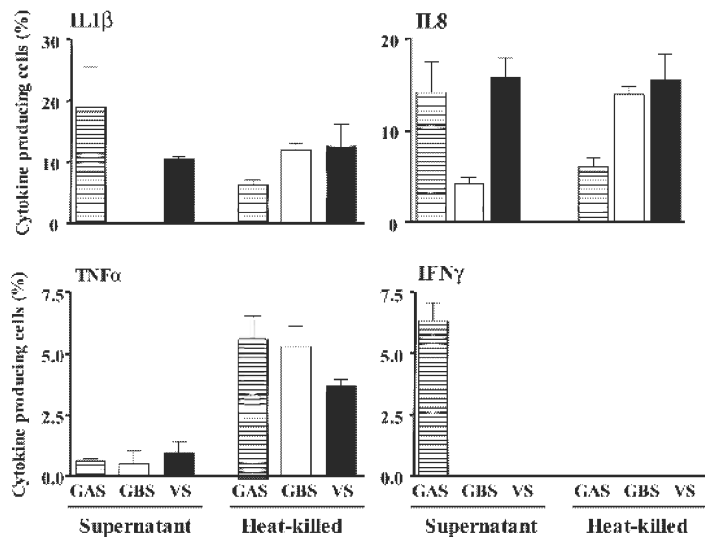


Figure 6. Cytokine induction profile of GAS, GBS, and viridans streptococcal isolates. PBMCs were stimulated with GAS, GBS, or viridans streptococcal (VS) culture supernatant or heat-killed bacteria. Cells were harvested and stained for IL1 β , IL8, TNF α , and IFN γ by immunohistochemistry.

IVIG AS ADJUNCTIVE THERAPY IN STREPTOCOCCAL TOXIC SHOCK SYNDROME

The central role for superantigens and their induction of cytokines, together with the reported correlation between lack of humoral immunity and invasive GAS disease, suggested that IVIG was a potential adjunctive therapy in these diseases. IVIG has been shown to contain antibodies that can neutralize a broad spectrum of streptococcal superantigens (Norrby-Teglund *et al.* 1998). Importantly, IVIG-therapy was associated with decreased mortality rates in STSS patients in two clinical trials, one observational cohort study and one placebo-controlled trial (Kaul *et al.* 1999; Darenberg *et al.* 2003).

Neutralizing activity against GAS superantigens in IVIG preparations (IV)

The majority of studies on immunoglobulin (Ig) adjunctive therapy in gram-positive sepsis and shock have focused on IgG; however, some studies have suggested that IgM-enriched IVIG preparations are superior in the treatment of neonatal and gram-negative septicemia, as compared with IVIG preparations containing only IgG (Schedel *et al.* 1991; Poynton *et al.* 1992). Therefore, we felt it was important to investigate the comparative neutralizing activity of polyspecific IgM, IgA, and IgG on GAS superantigens.

The ability of Ig preparations containing various concentrations of IgG, IgM, and IgA to neutralize the mitogenic and cytokine-inducing activity of GAS virulence factors was tested. Inhibitory activity against the superantigen SpeA and against GAS culture supernatant, containing a mixture of superantigens (SpeB and SpeF, but no detectable SpeA) and other extracellular virulence factors, was determined. Eight different Ig preparations with varying isotype composition were studied (table 1), and all, except IgA from colostrum, were found to inhibit the proliferative and cytokine-inducing capacity of GAS superantigens. Interestingly, Ig-preparations containing IgM and/or IgA were more efficient inhibitors of SpeA, than of culture supernatant, whereas the reverse was true for IgG preparations.

However, only serum IgA, but not IgA purified from colostrum, inhibited the activity of GAS superantigens. ELISA determination of levels of superantigen specific antibodies present in the various Ig-preparation showed that the noted variation in neutralizing activity was not related to the amount of specific superantigen antibodies measured by ELISA. This is in agreement with previous reports demonstrating lack

of correlation between quantity and quality of superantigen antibodies (Norrby-Teglund *et al.* 1994; Basma *et al.* 1999). We have not yet identified the reason why serum IgA but not colostrum IgA are inhibitory, but ongoing studies are addressing this. We are currently analyzing matched serum and colostrum samples collected from post-partum women to determine whether our previously tested IgA colostrum were obtained from women who lacked protective immunity to GAS superantigens due to lack of exposure to GAS.

Our data show that IgM and IgA are efficient in neutralizing the proliferative and cytokine-inducing capacity of GAS superantigens, and that purified IgA was more efficient than IgG in the neutralization of SpeA. The most efficient neutralization of SpeA was achieved by a preparation containing a mixture of IgG, IgM, and IgA. This is of importance, since previous studies have shown that it is rare to reach 100% inhibition of SpeA when using physiological concentrations of IVIG (Norrby-Teglund *et al.* 1996; Norrby-Teglund *et al.* 1996). This is in contrast to other GAS superantigens, which are completely inhibited at very low concentrations of IgG (Norrby-Teglund *et al.* 1996; Norrby-Teglund *et al.* 1996). Previous studies have shown that several different toxins can trigger STSS and that there may very well be more than one toxin involved in each patient affected (Kotb 1995). However, SpeA has been suggested to be a pivotal virulence factor in outbreaks of severe invasive GAS infections.

Another important observation of this study was that the *in vitro* neutralizing activity of the IVIG batch corresponded to the plasma neutralizing activity following administration of IVIG. A similar correlation between *in vitro* and *in vivo* activity was noted in the multicenter trial of IVIG in STSS (Darenberg *et al.* 2003), and together the data suggests that even small variations in neutralizing activity may be clinically important. This study also suggests that optimization of IVIG-therapy in invasive GAS diseases may be achieved by changing the type of Ig-preparation.

IVIG-therapy in GBS and viridans streptococcal sepsis?

There is strong support for the use of IVIG in GAS toxic shock syndrome, much less is known about its use in severe sepsis caused by other streptococcal strains. A decreased mortality rate following IVIG administration, 18% as compared to 39% in infants who did not receive IVIG, has been reported in infants with early onset GBS

disease associated with neutropenia (Friedman *et al.* 1996). One of the cases included in paper II had alpha-streptococcal shock associated with ARDS, and required ICU and ventilation support during the episode of ARDS, despite receiving steroids during one week previously. At this point, she was given IVIG (0.5 g/kg bodyweight, once daily) for 4 consecutive days and she showed a remarkable clinical improvement. After 48 hours the inflammatory parameters decreased, her lung function improved and she could leave the ICU after 10 days. We conducted *in vitro* experiments to assess the inhibitory activity of IVIG against GBS and viridans streptococcal sepsis isolates. Preliminary data indicate that the pro-inflammatory activity of GBS and viridans streptococcal isolates is inhibited by IVIG, but not to the same extent as GAS superantigen induced responses. Hence, IVIG may potentially be an efficacious adjunctive therapeutic strategy also for GBS and viridans streptococcal sepsis, but this remains to be proven in clinical and experimental studies.

CONCLUDING REMARKS

Sepsis and septic shock are serious manifestations, which can affect individuals of all ages. Since the late 1980s, a marked increase in the incidence and severity of invasive GAS infections has been reported worldwide. During the same period, a pronounced increase in incidence and severity of bacteremia caused by viridans streptococci in immunocompromised patients was also noted. The case fatality rate of severe sepsis has remained high despite improved modern medicine and prompt antibiotic therapy; thereby, underscoring the importance of novel therapeutic strategies in these diseases. The pathogenesis of severe sepsis has been shown to involve interaction between several host and bacterial factors resulting in excessive production of inflammatory cytokines that mediate the systemic symptoms of severe sepsis and septic shock. Increased knowledge of the interactive forces between host and bacterial factors in the pathogenesis of streptococcal sepsis has led to novel therapeutic strategies to attenuate these diseases. One of the more promising therapeutic strategies includes IVIG-therapy that has been shown to be efficacious in STSS, and potentially also in necrotizing fasciitis cases. To our knowledge, there are no published clinical studies of IVIG-therapy in viridans streptococcal sepsis, and only limited data on GBS infections. The remarkable clinical improvement noted in the one patient who received IVIG in paper III, in addition to our preliminary in vitro data, warrant further investigation of the use of IVIG in toxic shock caused by viridans streptococci or GBS.

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