Superantigens in Group A Streptococcus

Gene Diversity and Humoral Immune Response

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To Martin

All vetenskaps början är förvåningen över att tingen är som de är.

Aristoteles (384-322 f.Kr.)

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ABSTRACT

Group A streptococcus (GAS) is a strictly human pathogen that causes infections ranging from asymptomatic carriage to the highly lethal streptococcal toxic shock syndrome (STSS). GAS are classified according to the sequence of the variable 5' end of the *emm*-gene that encodes the surface associated M-protein. In the late 1980s, outbreaks of GAS infections with high rates of STSS were reported in several parts of the world, including Sweden. Superantigens (SAgs), a group of exotoxins, have been described as key mediators of STSS due to their capacity to polyclonally activate T-cells and induce a massive release of inflammatory cytokines. Previous reports have revealed that sera from STSS patients have lower capacity to neutralize this SAg-mediated immune stimulation and a higher prevalence of GAS isolates with specific *emm*-genotypes during disease outbreaks. The aims of this thesis were to analyse the protective antibody response mounted by the host against SAgs produced by the infecting GAS isolate and to characterise the isolates *emm*-genotypes and SAg gene profiles. The clinical material examined was collected from patients with STSS, sepsis, erysipelas, or tonsillitis in Sweden between 1986 and 2001. Both acute- and convalescence-phase sera were analyzed, along with the infecting GAS isolates. The 92 clinical GAS isolates examined were found to exhibit a high degree of genetic diversity in terms of the number and identity of their SAg genes. Isolates with a given *emm*-genotype could be divided into subgroups on the basis of their SAg gene profiles. Ten different SAg gene profiles were identified in the 45 *emm1* isolates examined; one of these ten was highly persistent, being observed in 22 isolates collected over 14 years. Two of the 11 known SAg genes in GAS, *smeZ-1* and *speA*, were more prevalent in the *emm1* associated profiles than in the SAg gene profiles of isolates with other *emm*-genotypes. Patients infected by GAS with the *emm1*-genotype were less likely to produce acute-phase sera that could effectively neutralize the T-cell mitogenicity induced by the infecting isolate's extracellular products (EP). Sepsis patients whose sera exhibited this lack of neutralizing ability were more prone to developing STSS. Most patients whose acute-phase sera did not effectively neutralize the EP from the infecting isolate lacked protective antibodies in their convalescent-phase sera despite having elevated ELISA titers. The results reported herein show that combining SAg gene profiling with *emm*-genotyping may be useful for tracking the spread of GAS clones in the community. It was also shown that a lack of neutralizing activity in convalescence-phase sera might be due to an inability of those patients to mount a protective immune response against SAgs produced by the infecting GAS isolate.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Grupp A streptokocker (GAS) också benämnd *Streptococcus pyogenes* är en bakterie som kan orsaka en rad olika infektionssjukdomar hos människan. GAS är den vanligaste bakteriella orsaken till halsfluss (tonsillit) och kan också orsaka lokala hudinfektioner som svinkoppor (impetigo) och rosfeber (erysipelas). Sällsynta men allvarliga sjukdomstillstånd kan också följa en GAS-infektion, t.ex. blodförgiftning (sepsis), vävnadsnedbrytande mjukdelsinfektion (necrotizing fasciitis) och toxiskt chock syndrom (streptococcal toxic shock syndrom (STSS)). Det finns flera sätt att karakterisera en GAS-bakterie (isolat). Ett sätt är efter vilken typ av M-protein de har på sin cellyta. M-proteinet anses vara en central faktor för GAS förmåga att infektera värden (virulens) och genom jämförelser av den variabla 5' delen av *emm-genen,* som kodar för proteinet, kan olika varianter urskiljas. I dag har över 190 olika *emm*-varianter identifieras bland GAS.

I slutet av 1980-talet rapporterades från flera delar av världen utbrott av GAS orsakad sepsis och STSS med hög dödlighet. STSS visar ofta en snabb progress med symtom som blodtrycksfall, organsvikt, blod koagulation och vävnadsskador. Liknande sjukdomstillstånd har setts i samband med *Staphylococcus*-infektioner som t.ex. tampongsjukan (TSS). En grupp av gifter (toxiner) som utsöndras av GAS och *Staphylococcus* och som har blivit förknippade med STSS och TSS kallas Superantigener (SAg). Ett superantigen är ett toxin som har förmåga att ospecifikt aktivera en onormalt stor andel av immunförsvarets T-hjälpar celler. Ett normalt antigen (kroppsfrämmande ämne) aktiverar ca 0,0001 % av en värds T-celler medan ett superantigen kan aktivera upp till ca 20 %. Aktiverade T-celler utsöndrar cytokiner som fungerar som aktiveringsmolekyler för andra immunförsvarsceller. Den massiva frisättningen av cytokiner som sker till följd av SAg-aktivering leder till en hyperaktivering av värdens immunförsvar vilket istället för att skydda individen kan orsaka t.ex. vävnadsskador, organsvikt och chock. Superantigenerna hos GAS förknippas också med de hudutslag som kan ses vid scharlakansfeber. Fram till i dag har 11 olika SAg identifieras hos GAS, varav tre är kodade av gener som är förknippade med kromosomalt DNA och de övriga sju med bakteriofag-associerat genom. Bakteriofag-associerade gener har i regel en mer instabil närvaro i bakteriens genom i jämförelse med kromosomalt-associerade gener.

Tidigare forskning har rapporterat om en högre förekomst av GAS-bakterier med specifika *emm*-varianter i samband med sjukdomsutbrott. T.ex. under utbrotten i slutet 1980-talet förekom GAS-bakterier av varianten *emm1* i

större utsträckning. Det har också rapporteras att STSS patienter generellt har en låg andel antikroppar i blodet som kan neutralisera massiv immunförsvarsstimulering orsakad av SAg. En antikropp produceras av immunförsvarsceller som svar på kontakt med ett främmande ämne (antigen) till vilken den sen kan binda och underlätta eliminering. Syftet med studierna i denna avhandling var att analysera patienters antikroppssvar mot den infekterande GAS-bakteriens utsöndrade SAg samt att karakterisera respektive GAS-bakteries *emm*-variant och genuppsättning av SAg, dvs. dess SAg-genprofil. Det kliniska materialet som studerats var insamlats i Sverige mellan 1986-2001 från patienter med STSS, sepsis, erysipelas samt tonsillit och innefattade både akut- och konvalescensfassera samt den infekterande GAS-bakterien.

De 92 GAS-bakterier som studerades uppvisade en stor genetisk mångfald gällande uppsättningen av SAg-gener. Även GAS-bakterier av samma *emm*-variant uppvisade olika SAg-genprofiler. Bland 45 GAS isolat av *emm*-variant 1 kunde tio olika profiler identifieras. Noterbart är att en av dessa profiler återfanns i 22 *emm1* bakterier insamlade under en 14 årsperiod. Akutfassera från patienter infekterande med GAS-bakterie av *emm*-variant 1 hade en lägre förmåga att neutralisera T-cells aktivering orsakad av den infekterande bakteriens extra cellulära produkter (EP) jämfört med akutfassera från patienter infekterade med GAS av andra *emm*-varianter. Patienter med sepsis vars akutfasserum uppvisade denna brist på neutraliserande förmåga utvecklade oftare STSS i jämförelse med de som hade en hög förmåga att neutralisera. De flesta patienter som saknade skyddande antikroppar mot T-cells aktivering orsakad av den infekterande bakteriens utsöndrade produkter saknade också detta skydd i sitt konvalescensfasserum. För patienter med serum som uppvisade skydd mot T-cells aktivering orsakad av utsöndrade SAg från det infekterande isolatet så var den neutraliserande förmågan ej verksam mot utsöndrade SAg från andra GAS-bakterier med en annan SAg-genprofil. Resultaten i denna avhandling visar att genom att kombinera *emm*-typing och bestämning av SAg-genprofil kan en spridning av specifika GAS-kloner i ett samhälle identifieras. Samt att för vissa individer kan det aktiverade antikroppssvaret, efter en GAS-infektion, inte blockera superantigenernas massiva aktivering av T-celler.

ABBREVATIONS

APC	antigen presenting cells
APSGN	acute post-streptococcal glomerulonephritis
ARF	acute rheumatic fever
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
emm	*emm* gene; M protein gene
EP	extracellular products
GAS	group A streptococcus
HLA	human leukocyte antigen
IL	interleukin
IVIG	intravenous polyspecific immunoglobulin G
KD	kawasaki disease
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MF	mitogenic factor
Mga, *mga*	multiple gene regulator (gene)
MHC	major histocompatibility complex
MLST	multilocus sequence typing
MMPs	metalloproteinases
MS	mass spectrometry
MW	molecular weight
NF	necrotizing fasciitis
OF	opacity factor
PBMC, PBMCs	peripheral blood mononuclear cell(s)
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
rMF	recombinant mitogenic factor
SAg, SAgs	superantigen(s)
S. aureus	*Staphylococcus aureus*
S. dysalactiae	*Streptococcus dysalactiae*
Sic, *sic*	streptococcal inhibitor of complement (gene)
SLO	streptolysin O
SLS	streptolysin S
SmeZ, *smeZ*	streptococcal mitogenic exotoxin Z (gene)
SOF	serum opacity factor
Spe, *spe*	streptococcal pyrogenic exotoxin (gene)
S. pyogenes	*Streptococcus pyogenes*
SSA, *ssa*	streptococcal superantigen (gene)
STSS	streptococcal toxic shock syndrome
TLR	toll-like receptor
TSST-1	toxic shock syndrome toxin-1

PAPERS IN THE THESIS

This thesis is based on the following papers, which will be referred to by their Roman numerals (I-III).

I. **Maripuu, L., F. Ekström, M. Norgren.** Characterization of mitogenic and DNase activity of recombinantly produced mitogenic factor from group A streptococcus. Manuscript.

II. **Maripuu, L., A. Eriksson, M. Norgren.** 2008. Superantigen gene profile diversity among clinical group A streptococcal isolates. FEMS Immunol Med Microbiol. **54**:236-244.

III. **Maripuu, L., A. Eriksson, B. Eriksson, K. Pauksen, S. Holm, M. Norgren.** 2007. Dynamics of the immune response against extracellular products of group A streptococci during infection. Clin Vaccine Immunol. **14**:44-51.

INTRODUCTION

1.1 Disease manifestations caused by GAS

Group A streptococcus (GAS) is a strictly human pathogen that is globally distributed and can infect people of all age groups. It can cause a wide spectrum of different manifestations, ranging from asymptomatic carrier states to life threatening streptococcal toxic shock syndrome (STSS). It is primarily spread via direct or indirect contact with aerosols from infected individuals, but food-borne outbreaks of GAS infection also occur (65, 74).

In general, between 3 and 6 % of the population are estimated to carry GAS asymptomatically in their respiratory tract; its prevalence is higher in children (12, 66, 219). Studies on children between the ages of 4 and 17 revealed carrier rates ranging from 8.4 to 15.9 %, with the highest rates being detected in less-developed countries (12, 58, 63, 129, 136, 219). Asymptomatic carriage of GAS can also occur in the skin, rectum and vagina, and is likely to function as reservoir of GAS bacteria in the population, spreading the bacteria to more susceptible individuals (56, 198). GAS is the major cause of bacterial pharyngitis and tonsillitis, having the highest prevalence in children and adolescents (22, 35). GAS can also cause other respiratory tract related infections, including otitis, pneumonia, and meningitis (88, 100, 152, 199).

Tissue infections caused by GAS are characterized according to the depth of the infection and primarily affect the extremities. Impetigo is a highly contagious localized infection of the epidermis, with GAS bacteria primarily associated with non-bullous impetigo, which gives rise to numerous small pimples surrounded by circles of reddened skin; the pimples burst, forming crusts. Non-bullous impetigo primarily affects children below school age (25, 35). Erysipelas is an infection primarily localized to the epidermis, dermis and cutaneous lymphatic tissue; it is characterized by sharply demarcated inflammatory reddish swelling. Cellulitis is a deeper infection of the subcutaneous tissue that lack clear distinction between infected and uninfected tissue (25). Necrotizing fasciitis (NF) is an infection that spreads rapidly in the subcutaneous tissue, progressing to both the epidermis and to deeper tissues such as fat and muscle fascia. Its clinical presentation often starts with severe local pain and swelling, accompanied by flu-like symptoms; the infected area becomes discolored and forms vesicles before turning necrotic (142). In a prospective population-based surveillance study conducted between 1992 and 1995 in Ontario, Canada, the incidence rate for NF increased from 0.085 to 0.4 per 100,000 individuals and year. Forty-six

percent of the NF patients were bacteremic and 47 % were associated with the presence of STSS (109).

Table 1. Case definition for Streptococcal Toxic Shock Syndrome (STSS)[a]

Definitive STSS case: criteria IA and II
Probable STSS case: criteria IB and II, with no other etiology identified
I. Isolation of GAS
A From a normally sterile site.
B From a non-sterile site
II. Signs associated with severe infection
A Hypotension
AND
B ≥ 2 of the following signs
1. Renal impairment
2. Coagulopathy (blood clotting disorder)
3. Liver involvement
4. Adult respiratory distress syndrome
5. A generalized erythematous macular rash
6. Soft-tissue necrosis

[a] Defined in 1993 by the working group on severe streptococcal infections (101). The table is partly adapted from (141).

Systemic GAS infections can cause sepsis and STSS. Sepsis can be defined as an infection of a normally sterile tissue, fluid or body cavity (documented or suspected), accompanied by a systemic inflammation response. In severe sepsis organ dysfunction is also seen (125). An epidemiological study of sepsis cases in the USA between 1979 and 2000 found that the mortality rate for patients with severe sepsis or septic shock without organ failure was around 15 %; for patients with three or more failing organs, the mortality rate was 70 % (135). A case definition for STSS was proposed in 1993 by "The working group on severe streptococcal infections" (Table 1) (101). STSS has a rapid progression and even when adequate intensive care treatments is available, its mortality rate ranges from 35 to 70 % (54, 70, 72, 170). The advent of full-blown STSS can be preceded by various different clinical presentations; tissue infections and sepsis are common, but it can also occur in previously healthy individuals for whom no portal of entry can be identified (56, 213). Two other potential systemic infections associated with GAS are scarlet fever and puerperal sepsis. Benign scarlet fever presents as a pharyngitis with a characteristic rash, known as "strawberry tongue", and desquamation of the skin. During septic scarlet fever, local suppuration and invasion of deeper structures are seen, while symptoms such as hyperpyrexia and neurological or cardiovascular complications are associated with toxic

scarlet fever (214). Puerperal sepsis is an infection of the endometrium and can occur in connection with childbirth or abortion. Common symptoms include fever, pelvic pain and abnormal vaginal discharges (133). Kawasaki disease (KD) is a systemic infection with clinical features similar to STSS, for which GAS has been suggested to be a causative agent. KD is described as an acute febrile mucocutaneaous lymph node syndrome that can cause serious complications including coronary aneurysms if left untreated (139).

Post-infectious complications that can follow a GAS infection include acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN). Both symptoms are regarded as delayed sequels to untreated pharyngitis, but APSGN can also follow an untreated skin infection. ARF is an autoimmune disease for which five major manifestations have been defined; inflammation of the heart (carditis), joints (arthritis), central nervous system (chorea), skin and/or subcutaneous nodules (erythema marginatum) (205). Of these, arthritis is the most frequent, while carditis is the most serious and is associated with mortality. The general symptoms of APSGN are dark urine, facial oedema, and hypertension. A transient decrease in the concentration of complement system factor C3 in the serum is a characteristic feature of this immune-mediated disease, and is important in its diagnosis (212) . The latency period for the development of ARF or APSGN is approximately three weeks following infection. Both ARF and APSGN are primarily seen in children (212, 232).

1.2 Epidemiology of GAS disease

The incidence of GAS infection is generally highest in less developed countries. For example, both respiratory tract and skin associated GAS infections (and their sequels such as ARF and APSGN) have been estimated to occur five to ten times more frequently in less developed countries than in developed countries (35). This generally higher incidence in the developing countries is probably due to lower access to medical care, poor hygiene and crowded living conditions (50). In epidemiological studies of GAS infections the disease manifestations are often designated as non-invasive or invasive, with invasive disease referring to GAS infections in otherwise sterile sites such as blood and CSF (101). However, for classification of infections the inclusion criteria are inconsistently applied. The incidence of invasive GAS infections the last two centuries has varied, both seasonally and over the years. In the 19th century, scarlet fever and puerperal sepsis were common GAS infections in the developed countries. Scarlet fever was considered a benign disease but epidemics of scarlet fever with high mortality rates of 5 to 25 % occurred in both Europe and the USA (108). In the 19th century, Swedish obstetricians reported that puerperal sepsis primarily affected

women suffering from malnutrition, although more widespread outbreaks were reported to be concomitant with outbreaks of scarlet fever. The case-fatality rate for puerperal sepsis was almost 50 % in Sweden and at its worst 20 % of delivering mothers died (96). As the use of antiseptic techniques became increasingly widespread among physicians and midwives during the late 19th and early 20th centuries, the incidence of both scarlet fever and puerperal sepsis declined (96, 108). In 1930 antibiotics such as the sulphonamides were introduced and markedly reduced the incidence and mortality rates of bacterial infections (108); in Sundsvall, Sweden, the introduction of antibiotics reduced the risk of contracting puerperal sepsis by 80 % (92). The incidence and mortality rates of severe GAS infections continued to decline thereafter, with the exception of a increase during the Second World War (188). In Norway, the incidence of invasive GAS infections was below 1.5 cases per 100 000 persons per year in the late 1970s (156). Overall, by the 1970s, severe GAS infections were much less common in the developed countries than had been the case in the late 19th and early 20th centuries.

In the mid and late 1980s, an upsurge of severe GAS infections was seen in several parts of the world including Europe, USA, Canada and Australia (57, 76, 79, 216, 232). The reports describe an increase in the number of bacteremia cases, outbreaks of acute rheumatic fever, and severe soft tissue infections associated with systemic toxicity and high mortality rates (79, 216, 232). In Norway, national surveillance of non-invasive and invasive GAS infections was established in the mid 1970s and it was noted that both non-invasive and invasive GAS infections increased considerably during the winter of 1987-1988. The incidence of non-invasive GAS infections rose by 60 % compared to the preceding decade and the frequency of GAS bacteremia increased from a mean of 30 cases per half-year to 84 cases in the first half of 1988 (137). In Sweden, an outbreak of GAS bacteremia occurred during the winter of 1988-1989, with the number of reported cases rising from 26 in January 1987 to 54 in December 1988 and 41 in January 1989 (218). These outbreaks of invasive GAS infections prompted more intensive monitoring of the situation (118). In Sweden, national surveillance of invasive GAS infections was initiated in 1988 (223). The monitoring of invasive GAS infections was made mandatory under Swedish laws concerning infectious disease control in July of 2004 (202). The worldwide enhanced surveillance during the last two decades has greatly enlarged the epidemiological data showing that the 1980s outbreaks were not isolated incidents (135). In Sweden, annual incidence rates of invasive GAS disease ranging from 1.8 to 3.0 per 100 000 inhabitants were observed in national surveillance studies conducted between 1987 and 2004 (54, 72, 218, 223). A shift in serotype distribution in both the invasive and non-invasive isolates

has been observed during periods with higher incidences of invasive GAS disease (54, 66, 196). The high prevalence of M1/*emm1* isolates during the outbreaks at the end of 1980s has persisted, although a high prevalence of additional serotypes such as *emm89* and *emm81* in Sweden, *emm28* and *emm89* in Denmark*, emm28* and *emm82* in Norway, as well as *emm28* and *emm84* in Finland has also been reported (54, 66, 148, 200).

1.3 Classification of GAS

Group A streptococcus (GAS), *Streptococcus pyogenes,* belongs to the family *Streptococcaceae* and the genus *Streptococcus*. Streptococcus isolates can be classified into *α-, β-,* or *γ- Streptococcus* on the basis of the haemolysis pattern they create on blood agar plates. Alpha streptococci give partial haemolysis, beta isolates give complete haemolysis, and gamma isolates do not cause lysis. GAS causes complete haemolysis and thus belongs to the *β-Streptococcus* subgenus. The name "group A streptococcus" originates from the classification of the *β-Streptococci* into 22 different serological groups (A-V). The method is based on the immunological properties of the cell wall polysaccharide, as described by Rebecca Lancefield in 1933 (121).

Detailed classification of pathogens facilitates both epidemiological studies and the identification of important virulence factors; GAS has been characterized and sub-typed using both serological and genotypic methods (Fig. 1). The surface-associated M-protein has frequently been used for sub-typing of GAS isolates. Serological typing with respect to the M-protein was introduced in 1928 by Rebecca Lancefield and is described as M-typing in the literature (120). A key limitation of the method is that it requires a type-specific polyclonal anti-serum, which is an expensive reagent to produce. During the 1990s, typing on the basis of the sequence of the 5' end of the gene encoding the M-protein was established and is referred to as *emm*-genotyping (11). Compared to M-serotyping, *emm*-genotyping is easier to perform, more accessible, and less time-consuming. Moreover, it has led to the identification of many new GAS subtypes. An *emm* sequence database has been established and over 190 different *emm*-types encompassing over 800 subtypes have been identified, which makes *emm*-genotyping one of the most extensive methods for subtyping GAS (38). A one-to-one relationship between the *emm*-genotypes and most of the existing M-types has been seen (103).

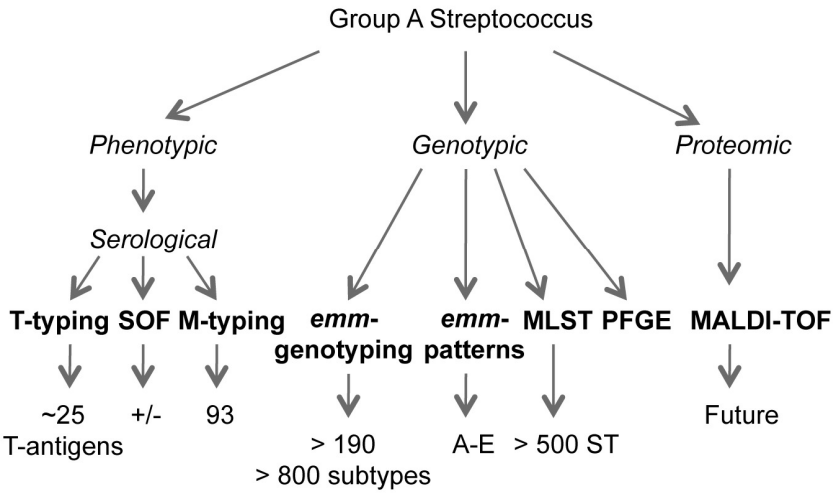

Figure 1. Methods for typing GAS.

Two additional classification methods that serve as useful adjuncts to M-serotyping and *emm*-genotyping are T-protein serotyping and the detection of serum opacity factor (SOF). Typing of the surface T-antigen was developed by Griffith in 1934s; more than 25 distinct serotypes have been identified to date (87). In addition, a GAS isolate can express more than one T-antigen type, resulting in complex T-agglutination patterns. This method can be used to further differentiate M and/or *emm*-genotypes (103). T-antigen forms pilus-like structures on the bacterial surface and has been proposed to function as an adhesion factor (150). The SOF test distinguishes between OF+ and OF- isolates; approximately half of all GAS isolates produce SOF (103). SOF is an apoproteinase that can induce the formation of large lipid particles in serum (49).

GAS isolates can also be sub-grouped on the basis of their *emm* patterns, i.e. the number of *emm* and *emm*-like genes. Many isolates carry one or two *emm*-like genes located immediately up- or down-stream of the *emm* gene. The *emm* and *emm*-like genes can be grouped into four gene subfamilies (SF1-SF4) on the basis of sequence differences near the 3' end. An *emm*-pattern is defined in terms of the isolate's number of *emm* and *emm*-like genes, their SF content, and their relative arrangement on the chromosome (20). Almost all isolates have one of five *emm* patterns (A-E) and a correlation between isolates' *emm*-patterns and tissue tropism has been identified (21). *emm*-patterns A to C are observed in throat isolates, pattern D is seen in skin isolates, and pattern E is found in both throat and skin isolates. Pattern E isolates are considered generalists and account for

approximately 50 % of all GAS isolates (21). The *emm*-pattern correlates with the *emm*-genotype in that isolates of a given *emm*-genotype usually exhibit the same *emm*-pattern (143).

Another method for sub-grouping GAS is multi-locus sequence typing (MLST). MLST is a genotypic classification method in which sequence differences in internal fragments from seven to nine species-specific housekeeping loci are used to distinguish between isolates. An MLST database has been established for GAS, and it appears that the MLST results for a given GAS isolate correlate strongly with its *emm*-genotype (67, 143). Analysis of the banding patterns generated by pulse-field gel electrophoresis (PFGE) of completely-digested chromosomal DNA from GAS isolates have proven to be an efficient tool for identifying clonal relationships between GAS isolates (19). A newly described method for typing GAS involves the use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to characterize the isolate's proteomic profile. Moura et al. have recently suggested that MALDI-TOF MS can be used to distinguish between invasive and non invasive GAS isolates (151).

1.4 GAS virulence factors

The clinical manifestations of GAS infections are numerous and so are the virulence factors expressed by the bacteria. Virulence factors can be defined as the substances produced by the pathogen that are involved in the initiation and maintenance of infections. They can be either cell-associated or secreted. The first complete sequence of a GAS genome revealed over 40 putative virulence genes (75). It has been shown that different GAS isolates have different panels of virulence genes, and of regulatory genes affecting their expression (23). A GAS clone often has several virulence factors that contribute in each step of the pathogenic process and may be capable of mediating several functions (Fig. 2).

1.4.1 Colonization and intracellular uptake

The pharyngeal mucosa and the skin represents the primary sites for GAS entry to the human host. GAS must extract nutrients from the environment to proliferate in saliva; a surface lipoprotein, maltodextrin-binding protein, has been shown to accumulate nutrition by binding maltodextrin in saliva to the bacterial surface (197). The attachment of GAS to host tissue can be mediated via several different adhesion molecules; the precise identity of the adhesion molecules produced by a given GAS isolate plays a role in determining its tissue tropism.

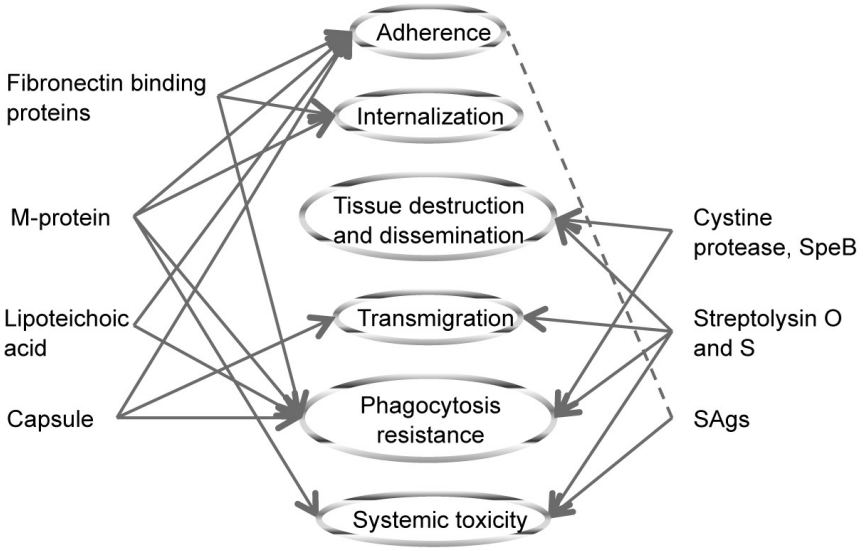

Figure 2. Examples of virulence factors in GAS with pleiotropic effects. Surface-associated virulence factors are listed on the left, secreted virulence factors on the right. The dashed line indicates one of the proposed functions of SAgs –localized overstimulation of T-cells to inhibit the functional activation of the humoral immune response against the infecting isolate.

GAS primarily binds to fibronectin, integrins, collagen, glycosaminoglycans, and the CD44 and CD46 receptors in the extracellular matrix and on the host cell surface. The most extensively studied adhesins in GAS are M-protein (171), the fibronectin binding proteins (F1, F2, and Fba) (47), pili (134), collagen binding proteins (114), lipoteichoic acid (LTA) (47), and hyaluronic acid capsular polysaccharide (51). Biofilm formation is a virulence strategy in bacterial colonization of the host and the colony's subsequent persistence. The presence of biofilm-like structures in skin sections from impetigo patients has been associated with GAS infections; the virulence factors suggested to be involved are the pili, M-protein, LTA, and streptococcal protective antigen (2, 48, 134). Interestingly, the presence of the GAS cysteine protease streptococcal pyrogenic exotoxin B (SpeB) has been negatively correlated with biofilm formation (61). Isolates of GAS have shown to differ in their tendency to form biofilms; an increased tendency to form biofilms is known to correlate with increased resistance to the host's defenses and antibiotics (45, 123).

GAS is primarily an extracellular pathogen but it has been proven capable of initiating its uptake into non-phagocytic human cells and of persisting in the intracellular environment in both human lung and skin cell cultures (17). The M- and F1-proteins have been shown to trigger the internalization of GAS into *e. g*. epithelial cells. They both bind to fibronectin and interact with

integrins on the host cell surface but the subsequent downstream signaling events in the host cells differ for the two groups of adhesins. The M-protein triggers a cytoskeletal rearrangement in the host cells that causes microvilli to be recruited around the bacterium, resulting in its uptake into phagolysosomes via a zipper-like mechanism (62). Interaction with host cells via the F1 protein leads to the formation of caveolae beneath the attached bacteria, resulting in large invaginations (174). Intracellular growth can be a way for bacteria to avoid clearance by phagocytes, antibodies and/or antibiotics. Intracellular GAS has been recovered from the tonsils of patients with pharyngeal infections that are recurrent despite ongoing antibiotic treatment (173).

1.4.2 Tissue destruction and bacterial dissemination

Tissue destruction and bacterial dissemination can precede the systemic spreading of a local GAS infection. GAS virulence factors associated with host tissue destruction include a secreted phospholipase A_2, SlaA, that hydrolyzes phospholipids (201), a secreted carboxylic esterase, SSE, that cleaves esters (238), the surface bound streptolysin S (SLS), which causes osmotic host cell lysis via the formation of transmembrane pores (55), and the SpeB that activates the host's matrix metalloproteinases (MMPs) and cleaves plasma kininogen to kinin, as reviewed by Chian-Ni et al (40). MMPs are known to degrade the host's extracellular matrix, and kinin is a potent proinflammatory molecule that increases the vascular permeability of the infected tissue. Strains with mutations in each of these four virulence factors exhibited reduced tissue destruction and bacterial dissemination in infected mice compared to the corresponding wild type strain (55, 131, 201, 238).

The spread of a GAS tissue infection is believed to be caused by the activation of the human serine protease plasmin. Plasmin derives from plasminogen, which is present in the plasma and enriched at the site of infection due to vascular leakage. Adhesion proteins such as the M-like proteins bind plasminogen to the GAS surface; the GAS streptokinase, Ska, can form an activated complex with the bound plasminogen proenzyme and this complex converts unbound plasminogen into the serine protease plasmin (229).

1.4.3 Invasion and phagocytosis resistance

GAS invasion can also occur through the epithelial cell layers without intracellular uptake or tissue destruction; this phenomenon has been connected to the presence of the capsule. The capsule has been shown to

Table 2. Antiphagocytic mechanisms employed by GAS

Virulence function	Mechanism	Mediating virulence factors
Prevent neutrophil recruitment	interfere with the chemoattractant IL-8, and the complement factor C5a	Cell envelope protease (SpyCEP), Streptococcal C5a peptidase (ScpA) (64), and Surface dehydrogenase (SDH) (224)
Protect against neutrophil capture	degrade the neutrophil extracellular traps	DNases such as Sda1 (33)
Prevent opsonization of the bacteria	interfere with opsonin factors in the complement system, and with antibody opsonization	surface adhesins such as the Fba-, M-, and M related- proteins (Mrps) (16, 46, 176), Capsule, M- and F1-protein, endoglycosidase, cysteine proteases IdeS and SpeB (1, 36, 42, 68, 147)
Prevent bacterial uptake into phagocytes	develop bacterial aggregates, masking the bacteria, and interfere with the phagocyte cytoskeleton	M- and F1-proteins and streptococcal inhibitor of complement (SIC) protein (60, 91, 192)
Trigger phagocytic cells death	lysis of the phagocytic cells, and induction of apoptosis	SLS, SLO, and SpeB (55, 116, 225)
Avoid bacterial clearance inside the phagocyte cells	inhibition of phagosome maturation, adaption to oxidative stress, resistance to antimicrobial peptides and lysozyme, and inactivation of the bactericidal LL-37 and the alpha defensins	M-protein (210), glutathioneperoxidase (GpoA) (29), D-alanylation of membrane bound LTA (115), SIC, and SpeB (78, 169)

mediate binding to CD44 on epithelial cells and to trigger cytoskeletal rearrangements and disruptions of the intercellular junctions that allow GAS to remain extracellular as it penetrates the epithelium (52). GAS isolates can vary greatly in their degree of encapsulation, and mucid isolates have been associated with a higher incidence of invasive infections (104). Invasion through the epithelial cell layers may also be an important mechanism for bacterial transmigration to tissue distant from the entry site. For example, in NF patients, the affected site may be distant from the proposed entry site. SLS is another virulence factor that is known to contribute to the translocation of GAS across the epithelial cell layers. An *in vitro* study by Sumintomo et al showed that SLS facilitates GAS invasion by degrading the epithelial intracellular junctions in conjunction with calpain, a host cystein protease (220). For bacteria to survive and continue to cause infection, they have to resist being killed by phagocytic cells. GAS bacteria possess several mechanisms that promote resistance to phagocytic clearance (Table 2) (117).

1.4.4 Systemic effects

Unbalanced immune responses due to uncontrolled cell activation and the release of elevated levels of inflammatory cytokines can generate systemic effects in the host such as vascular leakage and coagulation, followed by circulatory collapse and organ failure (165). The primary GAS virulence factors associated with excessive production of cytokines in the host are the superantigens (SAgs; see below). Other virulence factors with the potency to stimulate the release of inflammatory cytokines are the SLO, LTA and the peptidoglycan, but these generally have less pronounced effects than the SAg proteins (89, 206). It has also been suggested that the M1 protein in complex with fibrinogen induces systemic effects in the host by interacting with the B2 integrins and thereby activating the polymorphonuclear neutrophils (PMNs). Activated neutrophils can release heparin binding protein, an inflammatory mediator that induces vascular leakage in the host (90, 175). The M1/fibrinogen complex might thus be responsible for some of the symptoms seen in systemic infections such as STSS.

1.5 Superantigens in GAS

Superantigens are characterized by their ability to polyclonally activate T-cells. This activation is dependent on composition of the beta chain (TCR-Vβ) of the T-cell receptor and presentation of unprocessed SAgs by the antigen-presenting cells (77, 207). The presence of SAgs in GAS was first detected by protein-based methods through analysis of culture supernatants. However, with the establishment of convenient genome screening tools in

Table 3. Functional properties of GAS superantigens[a]

SAg	MW (kDa)	MHC II binding α/β chain	Human TCR-Vβ specificity[d]	P_{50} (h) (pg/ml)[e]	References
SmeZ-1[b]	24.3	-/+	2.1, **4.1**, 7.3, **8.1**	0.08	(81, 105)
SmeZ-2[b]	24.1	-/+	**4.1**, **8.1**	0.02	(183, 184)
SpeG[b]	24.6	-/+	**2.1**, 4.1, 6.9, 9.1, 12.3	2	(183)
SpeJ[b]	24.6	-/+	**2.1**	0.1	(6, 140, 181)
SpeA[c]	26.0	+/-	2.1, **12.2**, **14.1**, 15.1	?	(99, 177, 193, 233)
SpeC[c]	24.4	-/+	**2.1**, 3.2, 12.5, 15.1	0.1	(85, 124, 126, 191)
SpeH[c]	23.6	-/+	2.1, **7.3**, 9.1, 12.3	50	(3, 183)
SpeI[c]	26.0	-/+	6.9, 9.1, **18.1**, 22	0.1	(181)
SpeK/L[c]	27.4	-/+	**1.1**, 5.1, 23.1	1	(15, 98, 186)
SpeL/M[c]	26.2	-/+	**1.1**, 5.1, 23.1	10	(186, 203)
SpeM[c]	25.3	?	**1.1**, 5.1, 23.1	?	(203)
Ssa[c]	26.9	+/-	1.1, 3, 15	?	(149)

[a] The table is partly adapted from Proft et al.(182).
[b] Chromosomally encoded SAgs
[c] Prophage-associated SAgs.
[d] The major T-cell Vβ targets of the SAg proteins are shown in bold.
[e] P_{50}(h)=concentration needed for half maximum proliferation of human T-cells.

the 1990s, DNA-based methods such as polymerase chain reaction (PCR) have largely replaced protein-based screens. By the end of the 1990s, four SAgs had been identified: SpeA, SpeC, Ssa, and SmeZ (99, 105, 124, 149, 182, 183). The first complete genome screening of GAS was published in 2001, and in the four years between 1999-2002, seven new SAgs were identified (*spe-G, -H, -I, -J, -K/L, -L/M* and *–M),* bringing the total number of known SAgs in GAS to eleven (Table 3)(15, 181, 183, 203, 204). Two SAg genes, *speK/L* and *speL/M*, were identified on two separate occasions and therefore have two different designations. The double nomenclature for these genes is used in this thesis (15, 98, 186, 204).

Although the genomes of nine new GAS strains representing eight different *emm*-genotypes have been sequenced since 2002, no further novel GAS SAgs have been identified (13, 146). However, DNA sequencing of SAgs in clinical isolates has revealed new allelic variants of the *smeZ* gene (Paper II) (184). Forty-seven allelic variants of the *smeZ* gene have been identified; they mainly differ within mosaic regions correlated to surface-predicted epitopes (Paper II) (183, 184, 189, 228). Substitutions within these regions have not been shown to have a major impact on the protein's Vβ specificity or its mitogenic activity (184, 221). Allelic variation has also been observed in *speA*, and *speC* but to a lesser extent (163). The six allelic variants of the *speA* gene can be divided into two distinct lineages, lineage I (*speA-1, -2, -3* and, *-6*) and lineage II (*speA-4, -5*); variants within a lineage differ by

approximately one or two synonymous or non-synonymous amino acid changes (20). Similarly minor allelic variations have been seen in the four *speC* alleles (163).

The SpeB and SpeF proteins were first recognized as superantigens in GAS and were also shown to have proteinase and DNase activity, respectively (27, 102, 237). However, in 1994, the SAg activity detected for the natively purified SpeB protein was attributed to contamination of the sample with SmeZ (82). Further proof that SpeF, which is also known as the mitogenic factor (MF), is a DNase was obtained when the corresponding gene was shown to be identical to that for DNaseB, a well characterized DNase in GAS (209). The SAg activity of SpeF/MF was questioned in 2001 by Gerlach et al., who detected no SAg activity for this protein when natively purified (83). Results presented in this thesis indicate that while the SpeF/MF protein is indeed a DNase, it is not a superantigen (Paper I).

1.5.1 Superantigens and their structures

It has been reported that superantigen toxins are also produced by *Mycoplasma arthritis, Yersinia pseudotuberculosis*, *Yersinia enterocolitica* and *Staphylococcus aureus*. The superantigens of GAS are similar to these of *S. aureus* but show little or no homology to toxins from Gram-negative bacteria. Interestingly, viral SAgs have been detected but unlike secreted GAS SAgs, they are expressed on the bacterial cell (112). Other streptococcal species that harbour SAgs include *Streptococcus equi* and *Streptococcus dysalactiae*; recently, two SAgs (*speL/M* and *speM*) were detected in both GAS and *S. equi* isolates. Homologies between other SAgs in GAS and in *S. equi* and the *S. dysalactiae* subspecies *equisilis* have been observed; these inter-species similarities are probably due to horizontal gene transfer (4, 77, 186).

Superantigens expressed by *Streptococcus* and *Staphylococcus* are soluble non-glycosylated molecules that are around 25 kDa in size. Structurally, they consist of two globular domains, an N-terminal β-barrel domain and a C-terminal β-grasp domain with a long α-helix that extends through the centre of the molecule. The SAg structure is generally stable and resistant to denaturing conditions such as heat and acid (6, 185). While the SAgs share a common and highly conserved tertiary fold, they can differ considerably at the amino acid level. The amino acid sequence identities for SAgs in GAS are between 17 to 48 % and two conserved motifs useful for the identification of new SAgs have been identified (182). Aminoacid sequence alignments of SAgs from GAS and *S. aureus* have shown that some GAS SAgs are more closely related to the SAgs of *S. aureus* then to the other GAS SAgs (77).

1.5.2 The GAS genome, gene regulation and SAg expression

To date, 13 GAS strains representing ten *emm*-genotypes have been sequenced, revealing that the GAS genome consists of a single circular chromosome approximately 1.9 Mb in size (9, 13-15, 75, 86, 93, 146, 155, 203). The "core genome" accounts for around 90 % of the sequence, with the remaining 10 % consisting of variable regions. The variable regions include insertion elements and prophage-like elements or their remnants (8, 13). Virulence factors encoded by phages or phage-like elements account for a large proportion of the observed differences in the gene contents of different GAS isolates. Horizontal transfers of prophage elements between GAS isolates and also between different streptococcal species have been observed (8). For example, the genes encoding the SAgs *speL/M* and *speM* in GAS were first identified in group C streptococcus by screening an *S. equi* genome database (186). In addition to the prophage-like elements, some isolates harbour unique genetic material on integrated conjugative elements (ICEs), with genes encoding resistance against antibiotics such as tetracycline and erythromycin (13). Genes for putative or known virulence factors that are not related to bacteriophages have been found in 11 loci located within five chromosomal regions, generally accompanied by mobile genetic elements (145).

The first described network for the regulation of virulence genes in GAS was the multiple gene regulator (Mga). Mga activates the transcription of several virulence factors including the M protein, C5a peptidase, serum opacity factor (SOF), speB, and secreted inhibitor of complement (Sic). The expression of these genes decreases in response to environmental conditions as increasing osmolarity, low temperatures, and restricted availability of iron. Proteins regulated by Mga have been observed to be expressed during the exponential growth phase but not during the stationary growth phase (144).

The molecular mechanisms underpinning the regulation of SAg gene expression are not yet fully understood. However, transcription of the *speA* gene is repressed by a member of the RofA-like protein family, the Nra transcription regulator, which also regulates other virulence factors as fibronectin binding protein, F2, and collagen-binding protein, cpa (144, 180). Nra is expressed during the early stages of the stationary growth phase, as is *speA*. In a study of SAg gene expression during experimental infection of Cynomolgus macaques with GAS, increased *speA* transcription was seen during the later stages of infection, when GAS density was high (234). In the same study, transcription of the *smeZ* gene dominated in the acute-phase and continued to increase during subsequent stages. Conversely, the *speJ*

gene was primarily transcribed during the colonization-phase. Studies on the expression of SAgs have shown them to be significantly upregulated during infection or when bacteria are co-cultured with human cells, suggesting that host factors influence their regulation (31, 234). Interestingly, the up-regulation of *speA* following infection was found to persist during growth in *in vitro* cultures (111).

1.5.3 Influence on the host

SAgs have the ability to activate a large portion of an individual's T-cell repertoire and thereby induce a massive release of cytokines in the host. This release is linked to the SAgs' capacity to cross-link T-cells with antigen presenting cells (APCs) through binding to the T-cell receptors' (TCR) V-beta subunits and to the alpha and/or beta subunits of the MHC class II without prior uptake and processing. The generated trimolecular complex triggers the activation of both cell types. Since each SAg has specificity for several TCR V-beta subunits, a large number of T-cells can be activated, inducing a massive release of pro-inflammatory and anti-inflammatory cytokines (77, 207). The broad T-cell response generated by the superantigens differs from a conventional activation, in which the interaction and activation are highly specific, being restricted by both the MHC class II receptor and the processed peptide. There are fewer than 50 different Vβ regions in the human T-cell repertoire. SAgs can activate approximately 20 % of the host's T-cell population, whereas a conventional antigen activates approximately 0.001 % of the T-cells (18). This large initial T-cell activation can be followed by anergy and Vβ-specific deletions that could reduce the host's ability to produce protective antibodies against either both the SAgs or and the invading pathogen (182). SAg-specific T-cell anergy has been shown to persist for several weeks following the initial infection (5).

The broad activation of the immune cells by SAgs can generate a systemic release of pro-inflammatory cytokines such as IL-1β, TNF-α, and TNF-β and the T-cell mediators IL-2, and IFN-γ, generating symptoms including fever, hypotension and multi-organ failure in the host (77). Several lines of evidence indicate that SAgs are involved in the pathogenesis of STSS and NF. Notably, SAgs have been detected in plasma from STSS patients and in tissue from NF patients (167, 208). The ability of SAgs to stimulate immune cells has been observed *in vivo* in STSS patients, who experience an expansion of a specific set of Vβ-bearing T-cell (231). Patients suffering from STSS or NF have been observed to have levels of the IL-2, IL-6 and TNF-alpha cytokines that were significantly greater than those in patients with less severe invasive infections (160). Additionally, several studies (Paper III) (10, 71) have

identified a link between a lack of SAg-neutralizing antibodies and the development of STSS.

The stimulation of lymphocytes by SAgs also triggers the release of anti-inflammatory cytokines such as IL-4, IL-5 and IL-10. The relative response ratio of anti-inflammatory cytokines to pro-inflammatory cytokines during the early stages of sepsis has been shown to affect the severity of the disease (222). A comparison of IL-10 and IFN-γ levels in plasma samples revealed the ratio of these two species to be significantly lower in STSS patients than in patients with systemic meningococcal disease (26). The way in which the SAgs are presented depends on the HLA-allele expressed; this can influence the outcome of the invasive infection and has recently been shown to affect the host cytokine response (163). In a study of patients with invasive GAS infections conducted by Kotb et al., a correlation between disease severity and the patient's HLA-II haplotype was identified; patients with DRBI*15/DQBI*06 were protected against severe sepsis, while patients with HLA-II DRBI*14/DQBI*05 haplotype had a higher morbidity (113). Recently, Mohammed et al. showed that the SAgs presented by the protective HLA-II haplotype generated a higher IL-10/IFN-γ ratio than did the high-risk HLA-II alleles (157).

Another activation mechanism by which SAgs are thought to enhance the release of cytokines involves the ligation of MHC class II receptors on the APCs; this has been demonstrated in a mouse model. One of the GAS SAgs (SpeC) has been shown to interact with MHC class II molecules, forming a cross-linking complex consisting of two SpeC molecules and two receptors (126). In an *in vitro* study, the cross-linking of MHC class II molecules induced by a staphylococcal SAg (SEA) was found to increase the membrane expression of TLR4 receptors on monocytes (95). The translocation of Gram negative bacteria has been observed during STSS, and LPS on the surface of Gram negative bacteria are recognized by the TLR4 receptor; this could potentially exacerbate the host's pro-inflammatory response.

1.5.4 Treatment

Group A streptococci are susceptible to β-lactam antimicrobials such as penicillin, cephalosporins, and the carbapenems. No resistance to these β-lactam antimicrobials has yet been observed in GAS, which is remarkable in light of the general spread of antimicrobial resistance that has been seen in various bacterial species around the world (7, 107). However, resistance to other antimicrobials such as sulfonamides (Bactrim), tetracyclines (Tetracycline) and macrolides (Erythromycin and Clindamycin) is known in GAS. Penicillin is the generally recommended antimicrobial agent for

treating GAS infections because of its proven efficacy and safety, narrow spectrum, and relatively low cost (24, 230). Neverthless, penicillin treatment has been known to fail in some cases, especially in more aggressive GAS infections (215). Penicillin treatments are sometimes subject to an inoculum effect that causes the penicillin to lose effectiveness as the bacterial growth rate slows; this may be due to reduced expression of a protein critical for penicillin-binding (217). Macrolide antibiotics can be used in patients who are allergic to β-lactams; in such cases, the resistance pattern of the infecting isolate must be considered (24). For GAS infections such as STSS, NF, and myositis, the recommended antimicrobial treatment is a combination of the β-lactam antimicrobials with Clindamycin (165). Clindamycin is a protein synthesis inhibitor and a potent suppressor of bacterial toxin synthesis whose efficiency is not affected by the bacterial growth stages (84, 215). Moreover, Clindamycin can promote the phagocytosis of the infecting isolates by inhibiting the synthesis of the protective virulence proteins such as the M-protein, (80). Because macrolide-resistant GAS isolates exist, Clindamycin should be used in conjunction with β-lactam antimicrobials (165).

Acute-phase sera from STSS patients have been observed to contain low levels of antibodies that can neutralize SAg-induced lymphocyte mitogenicity. This suggests that treatment with intravenous immunoglobulin (IVIG) could be beneficial (Paper III) (71, 185). IVIG has been shown to neutralize the mitogenic activity induced by GAS cell cultures and recombinantly produced SAg proteins in several *in vitro* studies (110, 161, 162). However, it has been observed that different IVIG batches can differ in their ability to neutralize the T-cell mitogenic activity of individual SAg proteins (195). Nevertheless, IVIG therapy has been reported to have beneficial effects in several STSS case reports (41, 119, 162, 178) and in a Swedish multicenter placebo-controlled trail of the clinical efficacy of IVIG treatment for STSS (53). In this latter study, IVIG-treated patients exhibited reduced mortality rates and a significant improvement in organ function compared to the placebo group.

At present, no vaccine against GAS infection is available. Several approaches have been tested, including mucosal vaccines containing conserved regions of GAS surface proteins such as the M-proteins, the C5a peptidase or the fibronectin-binding proteins. Multivalent vaccines prepared using type-specific epitopes of prevalent M-proteins are under development, and a 26-valent recombinant M protein vaccine has reached phase I/II clinical trials (211). The primary complications that hinder the development of a functional vaccine are the high diversity of the surface epitopes seen in GAS isolates and the necessity to avoid cross-reactions with human tissue.

AIMS OF THE STUDY

Superantigens (SAgs) are extreme modulators of the humoral immune system and have been found to play an important role in the rapid systemic progress of streptococcal toxic shock syndrome (STSS) caused by GAS. The aims of the studies described in this thesis were:

– to characterize the biological functions of the mitogenic factor (MF), which is present in almost all GAS isolates analyzed.

– to study the distribution of SAg genes in clinical GAS isolates.

– to analyse the development of the protective humoral immune response to SAgs in patients with different clinical manifestations of GAS infection.

METHODOLOGICAL CONSIDERATIONS

3.1 The clinical material

The clinical material included in this thesis was collected from patients infected with GAS and obtained from various hospitals in Sweden between 1986 and 2001. Patients with both invasive and non invasive GAS infections are represented, and the clinical material includes both blood samples from each patient and the corresponding GAS isolates. Some of this clinical material had been examined in previously-published studies (69, 71, 73, 94, 158, 166, 168).

The study described in paper II examined 92 clinical GAS isolates that forfilleds the following criteria: both the isolates and the corresponding acute and/or convalescent-phase sera were available, along with defined diagnoses for all of the patients. Clinical GAS isolates were collected from patients with STSS (30), sepsis (24), erysipelas (25), and tonsillitis (12). For one isolate, the patient's symptoms had not been recorded. Twenty-eight *emm*-genotypes were detected, nine of which were represented in two or more isolates (*emm1, emm28, emm2, emm8, emm12, emm66, emm4, emm75, and emm89*) (Table 4).

The samples included in paper III were selected according to the availability of both acute- and convalescent-phase sera, infecting GAS isolate and data on the patient's symptoms, treatment and course of disease (Table 5). The selection criteria were chosen to make it possible to determine the patient's humoral immune response to the infecting GAS isolate before and after the current infection. The patients were diagnosed by physicians following standard criteria (25, 101). For some patients, part of the disease information was collected retrospectively in collaboration with a physician. In total 75 patients satisfied the study criteria (Table 5). Nine extra isolates were added to the study and used to determine the ability of patient sera to neutralize the mitogenic activity of extra-cellular products (EP) from different GAS isolates.

Table 4. Clinical GAS isolates discussed in paper II

	Collection Year												
Genotype	-86	-87	-88	-89	-90	-91	-92	-93	-94	-95	-96	-01	No.
emm1		1	5	28	5			1	2	2		1	45
emm2	1			2							1		4
emm3			1										1
emm4						1					1		2
emm6									1				1
emm8				3		1							4
emm12				2		1		1					4
emm14							1						1
emm19			1										1
emm22					1								1
emm28				1	4			1					6
emm36			1										1
emm41	1												1
emm44				1									1
emm49				1									1
emm58		1											1
emm66			1	1	2								4
emm68				1									1
emm73				1									1
emm75					1				1				2
emm81			1										1
emm82					1								1
emm84								1					1
emm85				1									1
emm89					1	1							2
emm91					1								1
emm93			1										1
emm100								1					1
No.	2	2	11	42	16	4	1	5	4	2	2	1	92

The onset of disease was considered to have occurred on the first day the patient showed symptoms, which is a subjective measurement. In cases where the first day of symptoms was not reported the first day of admission to hospital was considered as day one of the disease. Acute phase sera were required to have been collected within seven days of the onset of disease; this criterion was chosen so as to minimize the likelihood that the acute serum samples would contain newly-produced antibodies against the infecting isolate. The convalescence-phase serum were divided into three groups on the basis of the days passed since onset of disease: early convalescent-phase

sera were collected between days 10 and 75, intermediate convalescent-phase sera between days 114 and 400, and late convalescent-phase sera between days 696 and 1512. The clinical isolates were sampled from the throat or nasopharynx, skin lesions, skin biopsies, ulcers, and blood. The risk of isolating a GAS species other than that which caused the disease was considered minimal for the isolates from blood cultures because blood is normally a sterile site. Conversely, the throat is a non-sterile site and can thus carry non-symptomatic GAS species (219). It would theoretically be possible to isolate a GAS species that did not cause the pharyngeal symptoms if dealing with a patient who was a non-symptomatic carrier of GAS and then became infected by another isolate. However, in such cases, the symptomatic GAS isolate would be expected to be substantially more abundant than the asymptomatic one, and would thus be more likely to be isolated. The GAS isolates from the erysipelas patients were collected from skin lesions for 18 of the 21 patients, and from the nasopharynx for the other three. Norrby et al. found that isolates from erysipelas patients' lesions were indistinguishable from those originating from the patients nasopharynx in terms of serotype, toxin production, and *emm*-pattern (168).

Table 5. Clinical material discussed in paper III

	Diagnose			
Number of	Tonsillitis	Erysipelas	Sepsis	STSS[a]
patients (mean age)	11 (18)	21 (59)	20 (46)	23 (60)
acute sera day 0-7	11	21	20	14
early sera day 10-75	11	20	13	10
intermediate sera day 114-400		3	3	1
late sera day 696-1,512			9	4

[a] Nine of the STSS patients received immunoglobulin treatment (IVIG and/or plasma); data on their sera are not shown in the table.

3.2 MF protein production

In paper I the MF protein was cloned in an expression vector and introduced into *Escherichia coli* to avoid contamination by other GAS SAgs. Because the addition of a tag (e.g. GST or 6xHisC) to facilitate purification can interfere with protein folding and thus stability, MF was purified by ion exchange chromatography.

3.3 PCR analyses

To optimize DNA yield and purity in paper II, three different DNA extraction methods were compared in parallel. These were the DNeasy Tissue kit (Qiagen Nordic, Stockholm, Sweden), the Blood tissue Genomic Mini kit (VIOGENE, Umeå, Sweden) and a glass milk extraction method described by Boom et al (28). A total of nine different GAS isolates were used in the analysis, and the colonies picked in the different assays originated from the same single spread on a blood agar plate. The highest DNA yield was obtained using the Blood tissue Genomic Mini kit (average of 40 ng/µl between experiments), followed by the DNeasy Tissue kit (19 ng/µl), and the glass milk extraction method (6.5 ng/µl). However, the DNeasy Tissue kit gave the highest sample purity and also the most consistent yields across multiple experiments. Since the presence of PCR-inhibiting substances can interfere with the amplification process and thus produce false negatives, we used the DNeasy Tissue kit for extracting DNA from the patient isolates (34). For the gene amplification process, primers were designed to yield a product whose size was similar to that of the full-length gene to be amplified. For each primer pair, the annealing temperature was estimated and a range of annealing temperatures within a ten degree interval around this estimate was examined. The use of an overly low temperature could result in unspecificity and mispriming, while too high a temperature could prevent primer annealing and thus PCR amplification (59). The temperature that gave the highest yield and specificity, as judged by agarose gel electrophoresis with EtBr staining was chosen. As a positive control for the amplification of each target gene, DNA from an isolate for which the identity of the resulting PCR product had been confirmed by sequencing was used. All sequencing analysis were performed in triplicates. To exclude the interference of PCR inhibiting substances in the DNA samples, PCR amplification of the *speB* and *mf/speF* genes, was used as a positive control. Isolates that gave negative PCR results during the analysis for *speH* and *speI* or *speL/M* and *speM* were tested by Southern blotting to confirm the absence of the gene. Each gene was amplified separately. This enabled the use of annealing temperatures optimized for each specific target gene. The optimal annealing temperatures for our selected primers varied between 44.0 and 59.1 °C.

3.4 Neutralization assay

In paper III the ability of patient serum samples to neutralize the lymphocyte mitogenic activity induced by the infecting isolates was tested using peripheral blood mononuclear cells (PBMCs) from healthy blood donors and ethanol precipitation of the culture supernatants from infecting isolates

grown in Todd-Hewitt broth for 16 h at 37◦C. The ethanol precipitate would be expected to contain the extracellular products (EP) expressed by the infecting isolate, including the superantigens (168). The importance of using the EP rather than a single purified SAg when testing the ability of patient serum to neutralize the lymphocyte mitogenicity of infecting isolates was shown by Basma et al., who found that some patients with high levels of neutralizing activity against a single SAg, SpeA, had low titers against the more complex mixture of superantigens in the isolate's EP (10).

Lymphocytes from six buffy coats were used in the neutralization assay. To exclude potential effects related to the possibility that PBMCs from different donors might respond differently, all serum samples from individual patients (and the corresponding EP) were tested in the same batch of experiments. For the same reason, the order in which the samples from the different patient categories (each of which represented several different *emm*-genotypes) were tested was varied randomly in each experiment. All EP were mitogenic at a dilution of 1/1,000 (titration experiments were conducted at dilutions between 1/500 and 1/5000). The sera and EP concentrations used in the assay were chosen on the basis of titration assays using human gamma globulin and several sets of EP samples. At the EP dilution used in the assay (1/100), a gamma globulin concentration of 82.5 µg/ml (corresponding to 5 % human sera) resulted in almost 100 % neutralization of lymphocyte stimulation, while a gamma globulin concentration of 16.5 µg/ml (corresponding to 1 % sera) achieved only 50 % neutralization. Five replicates were performed in all experiments. The neutralizing activity of a patient's serum was assessed by comparing the lymphocyte proliferative response caused by the infecting isolate's EP in the presence of the serum to that obtained in the presence of fetal calf serum. The fetal calf serum, which was used as a negative control, does not contain antibodies against streptococcal SAgs. The neutralizing activity of the patient serum against EP-induced PBMC proliferation was calculated as 1 – ((cpm of EP + sera)/cpm EP) x 100). Patients whose sera resulted in less than 50 % inhibition were categorized as low responders; those whose sera gave more than 50 % inhibition were categorized as high responders. This categorization scheme was chosen on the basis of the observed distribution of the activity levels of all the sera examined; most serum samples exhibited either very low (< 20 %) or very high (>70 %) inhibition (Paper III).

RESULTS AND DISCUSSION

4.1 SAgs identified in GAS

Each of the 92 clinical GAS isolates examined harboured between two and seven of the 11 known SAg genes; the chromosomally-encoded *speG* was present in all isolates (Paper II). This variation in the SAg gene content is consistent with previously-reported findings from studies in which all 11 SAg genes were screened for (32, 44, 127, 128, 148, 190). To date, only one GAS isolate with no SAgs has been reported; the absence of SAg genes in this isolate was confirmed by PCR screening of the eleven SAg genes and in a proliferation assay using human PBMCs and the supernatant from the isolate in question (127). Various large collections of clinical GAS isolates have been screened around the world; the fact that only one isolate has been found to be devoid of SAg genes indicates that there is a strong positive selective pressure on this virulence function in GAS (130, 132, 153, 186, 194).

Paper II also describes the sequencing of the *smeZ* genes; 22 different allelic variants were identified, including 14 novel alleles (*smeZ*- 26-30, and 40-48). The nucleotide sequences for the 14 novel *smeZ* alleles revealed non-synonymous sequence substitutions, primarily located within previously—identified mosaic regions. Substitutions within these regions have been shown to have no effect on the functions of the encoded proteins but to generate new antigenic variants that are not recognized by neutralizing antibodies in previously infected individuals (184, 221). This observed variability in the amino acid composition of the *smeZ* alleles illustrates the antigenic selection of novel variants in relation to herd immunity. While a particular *smeZ* allelic type was not exclusive for one *emm*-genotype, it was generally true that isolates of the same *emm*-genotype harboured the same *smeZ* allele. This might be due to an immune system-related selective pressure acting simultaneously on these two highly variable virulence genes.

When evaluating the results of studies on the involvement of SAg in GAS disease, it is important to consider the number of SAgs examined in each investigation. Many previous studies on SAg gene distribution focused on only a few of the 11 SAgs (130, 132, 153, 186, 194). Furthermore, the SpeB and SpeF/MF GAS exotoxins, (a protease and a DNase, respectively) are often included even though their roles as superantigens have been strongly questioned (Paper I) (82). The results described in this thesis demonstrated that the SAg lymphocyte mitogenicity assigned to native and recombinant purified MF protein in the early 1990s was probably due to contamination of

the purified MF samples with other mitogenic factors (Paper I) (164, 226, 237).

The contaminant in the purified MF that caused it to be incorrectly assigned as an SAg might have been the SmeZ-1 superantigen, since SmeZ-1 and the MF protein have similar molecular weights and isoelectric points, making them difficult to separate by the native purification methods such as chromatography and isoelectric focusing used in the studies discussed above. The SmeZ-1 protein is a very powerful mitogen, stimulating PBMCs at concentrations of 0.08 pg/ml; notably, the Oslo-85 GAS isolate, from which the MF protein was natively purified by Norrby-Teglund et al. belongs to the M1 serotype that probably encodes the smeZ-1 allelic variant of the *smeZ* gene (Paper II) (183). Another observation consistent with Sme-Z1 contamination being responsible for the misassignment of the MF protein as an SAg is that the TCR Vβ specificity of the supposedly "purified" MF (Vβ-2, -4, -8, -15, -19) was very similar to that of SmeZ-1 (Vβ-2.1, -4.1, -7.3, -8.1) (164, 183). The mitogenic activity attributed to the recombinant MF studied by Toyosaki et al. is also questionable due to the high concentration of MF (10 μg/ml) that was needed to induce PBMC proliferation. The use of recombinant proteins is associated with an increased risk of lipopolysaccharide (LPS) contamination (226). LPS, a glycolipid component of the outer membrane in *E. coli*, can induce PBMC proliferation at a concentration of 10 ng/ml (39). It should be noted that no results supporting the earlier findings that MF exhibits mitogenic activity have been published. However, there are two publications where MF was reported to not exhibit a mitogenic activity similar to a SAg, one on natively purified MF protein by Gerlach et al and in paper I of this thesis on recombinant MF protein (83). In our study, recombinant MF (rMF) was produced without a purification tag in order to maximize the likelihood that the expressed protein would fold correctly. The rMF protein was purified from disrupted expression cells both as soluble proteins and as refolded proteins from the inclusion bodies. Both the soluble rMF and the refolded rMF exhibited DNase activity (Paper I). The other SAgs identified in GAS have been successfully purified in *E. coli*, demonstrating that recombinant GAS proteins can be expected to exhibit SAg activity if the native protein is a SAg (182).

The MF protein's DNase activity is well established and was first proposed in 1997 by Iwasaki et al., who demonstrated that both natural and recombinant MF protein exhibit heat-resistant nuclease activity (102). In 2000, the gene encoding MF was shown to be identical to the DNase B gene (207). No other GAS DNases have been shown to have mitogenic effects on human PBMCs. For example, a recombinant version of the phage-encoded DNase Spd1 showed no ability to stimulate human PBMC in a proliferation assay (30). To

conclude, the results discussed above indicate that MF is a DNase but is not a SAg, and it is reasonable to assume that previous reports of mitogenic stimulation of PBMCs are attributable to SAg contamination in the case of native purified MF and LPS contamination in the case of recombinant MF. It is thus misleading to call this widespread chromosomally-encoded GAS protein "mitogenic factor"; "streptococcal DNase B" (Sda B) would be preferable.

4.2 Distinguishing between GAS isolates using their SAg gene contents

GAS isolates are often characterized on the basis of their *emm*-genotype. Several of the STSS outbreaks around the world since the late 1980s have been attributed to isolates of the *emm1* genotype (43, 56, 94, 97, 137, 227, 235). The correlations between *emm1* isolates and outbreaks of STSS were generally identified on the basis of an increased prevalence of this genotype in the outbreak areas. In light of the connection between SAg function and STSS symptoms, knowledge of the isolates' SAg gene content could be valuable for detecting unique SAgs within STSS isolates and for further distinguishing between clinical isolates.

The 11 GAS SAgs can theoretically be combined in 2048 different ways, giving 2048 different SAg gene profiles. We have shown that SAg gene profiles can be used to complement established methods for typing GAS, such as *emm*-genotyping, in order to further distinguish between GAS isolates (Paper II). In our study of 92 GAS isolates, 38 different SAg gene profiles were identified, along with 28 different *emm*-genotypes (31 if one distinguishes between different *emm*-subtypes). By combining *emm*-genotype data with SAg gene profiles, it was possible to define 45 different GAS isolate subgroups, or 49 if taking *emm*-subtypes into account (Paper II).

4.3 SAg gene distribution

The most common SAg genes identified in the SAg gene profiles in paper II were *speG* and *smeZ*, which occurred at frequencies of 100 % and 84 %, respectively. The least common SAg genes identified in the profiles were the *speL/M* and the *ssa* genes, which were present in 11 % and 13 % of the profiles, respectively (Paper II). The prevalence of the other seven known SAgs in GAS varied between 24 % and 61 %, depending on the gene (Table 6). This high frequency of the *speG* and *smeZ* genes, and the low frequency of the *speL/M* and *ssa* genes is consistent with results reported from other studies (44, 148, 189).

Table 6. Distribution of SAg genes within profiles

emm-type	No. of SAg profiles[a]	Percent of individual genes within the SAg gene profiles											
		smeZ	*smeZ-1*	*speG*	*speJ*	*speA*	*speC*	*speH*	*speI*	*speK/L*	*speL/M*	*speM*	*ssa*
All	38	84	29	100	39	37	61	34	29	26	11	24	13
emm1	10	100	100[b]	100	70	90[b]	60	20	20	10	0	20	0
None emm1	29	79	3	100	31	17	62	38	31	31	14	24	17

[a] One profile was present in both *emm1* isolates and isolates of other genotypes.
[b] The distribution of these genes in the *emm1*-associated profiles differed significantly from profiles in isolates of other *emm-geno*types.
Note: *emm*, M-protein coding gene; SAg, superantigen; *smeZ*, streptococcal mitogenic exotoxin; *spe*, streptococcal pyrogenic exotoxin; *ssa*, streptococcal superantigen.

The co-occurrence of specific combinations of the phage-associated SAg genes has been highlighted in several reports on the SAg content of GAS. The tendency of certain genes to occur together is typically explained by suggesting that some phages encode multiple SAg genes. Also, if it is difficult for specific combinations of phages to integrate with the core GAS genome, it would be rare for certain SAg genes encoded by different phages to coexist (44, 181, 183, 186, 204). For the phage-associated *speI* and *speH*, identified in tandem on the phage 370.2 in the *emm1* strain SF370, presence of one or both of the genes was equally common in the SAg gene profiles of our study and also noted by others (Paper II) (75, 189). A similar co-existence has been noted for the *speL/M* and *speM* genes, identified in tandem on phage phi speLM in the *emm18* strain MGAS8232. The detection of *speH*, *speI,* and *speM* as single genes in 16 %, 11 %, and 13 %, respectively, of the SAg gene profiles in our study could be due to the loss of the corresponding adjacent gene segments during the integration of the phage into the genome (Paper II). Interestingly, seven of the possible combinations of specific phage-associated SAg genes were not observed in any of the detected profiles (Paper II). However, six of these seven combinations have been observed in other studies on the SAg gene profiles of GAS isolates; the only combination that has never been observed to date is that of the *speL/M* and *ssa* genes (Paper II) (44, 148). It should be noted that one SAg gene profile contained ten of the 11 known SAg genes, lacking only the *speL/M* gene. Since the *speL/M* gene has been shown to be adjacent to the *speM* gene on the phi speLM phage and the *speM* gene was present in the profile that lacked only the *speL/M* gene, it is evident that all of the phages known to carry SAg genes should in principle be able to co-exist on the GAS genome. This finding highlights the uncertainty associated with interpreting

epidemiological data in terms of the size of the dataset required in order to draw reliable conclusions, especially when considering the absence of a particular gene.

4.4 SAg gene profile distribution within *emm*-genotypes

It was found that the primary differences in the SAg gene profiles of isolates with the same *emm*-genotypes involved the addition or loss of one or two phage-encoded SAg genes (*emm8*, *emm28*, *emm66,* and *emm89*). The *emm1* and *emm2* genotypes exhibited a greater degree of variation in their SAg gene profiles, with differences in up to seven of the SAg genes. This greater variation in the *emm1* and *emm2* isolates is probably due to that these isolates have been collected over longer periods of time and has been represented by a comparatively large number of isolates (Paper II).

Forty-five *emm1* isolates were examined in this study, making *emm1* the most common *emm*-genotype and that with the highest number of identified SAg gene profiles. No correlation between the SAg gene profiles of the *emm1* isolates and the year of their isolation could be identified. For instance, the *emm1* isolates obtained in 1989 exhibited nine different profiles (Paper II). Ten different SAg gene profiles were observed in the *emm1* isolates, with SAg gene counts ranging from three to seven; all of them contained the *speG* and *smeZ-1* genes. In addition, the *speA* gene was identified in nine of the ten profiles. The *speA* gene and allelic variant 1 of the *smeZ* gene were both significantly more common in the *emm1* isolates than in the SAg gene profiles of isolates with other *emm*-genotypes (Table 6). The *speA* gene was only seen in 3 % of the other *emm*-genotype isolates; the *smeZ-1* gene was present in 17 % (Table 6) (Paper II). The diversity in the SAg gene profiles of the *emm1* isolates showed that there were multiple *emm1* clones circulating during the outbreak of invasive GAS infections in Sweden in the late 1980s and early 1990s.

The 47 non-*emm1* isolates examined in this work represented 27 different *emm*-genotypes with 29 different SAg gene profiles. Of these *emm*-genotypes, *emm2*, *emm*4, *emm*8, *emm*12, *emm*28, *emm*66, *emm*75, and *emm*89 were represented by two or more isolates. All but three (*emm*4, *emm*12, and *emm*75) of these groups could be further sub-grouped on the basis of their SAg gene profiles. To evaluate the variation in the SAg gene profiles of the isolates from non-*emm1* genotypes, a larger dataset was constructed by combining our data with the results of Common et al. and Meisal et al., who also screened for all 11 known SAg genes in clinical GAS isolates (44, 148). Many of the clinical isolates examined by these authors were of the *emm12, emm28,* and *emm1* genotypes, and the diversity of the

emm28 and *emm12* isolates' SAg gene profiles was comparable to that observed for the *emm1* isolates examined in the work reported in this thesis. In total, the expanded dataset contained results for 52 *emm12* isolates with five different SAg gene profiles and 71 *emm28* isolates with seven different SAg profiles.

Dominant SAg gene profiles were identified for the *emm1, emm12, and emm28* genotypes; SAg genes that were common in multiple non-dominant profiles for a given *emm*-genotype were also present in the corresponding dominant profile. This could be taken to indicate that the dominant SAg gene profile for each *emm*-genotype corresponds to a successful clone whose daughter cells can gain or lose SAg genes to give the related minor profiles. This is consistent with the general exchange of phage-associated virulence genes that has been shown to occur between GAS isolates and also between GAS and other streptococcal species (8, 155, 186).

4.5 The identification of dominant and stable subgroups

While significant clonal variation was identified within the *emm1* isolates collected during 1989, a stable subgroup of isolates with the *emm1* genotype and SAg gene profile C (*speA, speG*, *speJ*, and *smeZ-1*), was identified in 22 of the 45 *emm1* isolates collected over a period of 14 years (Paper II). Common et al. recently reported that this SAg gene profile was both present and over-represented in clinical *emm1* GAS isolates from Australia, being observed in 27 of 29 *emm1* isolates; Meisal et al. reported similar findings in Norway, with this SAg gene profile being observed in 33 of 37 *emm1* isolates (44, 148). In addition, in a study where six GAS isolates were screened for all the 11 known SAg genes, three *emm1*-genotypes encoding *speA, speG*, *speJ*, and *smeZ-1* were identified (32). The stability of this genotype in clinical GAS isolates from diverse geographic areas over a period of approximately 21 years indicates that there is a strong selective pressure acting to maintain it in the circulation.

By combining our experimental data with that of Common et al. and Meisal et al., we were also able to identify dominant SAg gene profiles for *emm28* and *emm12* isolates collected over long periods of time and across a large geographic area (Paper II) (44, 148). The dominant profile in the *emm28* isolates was observed in 45 isolates of 70 that were collected over approximately 17 years. For the *emm12* isolates, the dominant profile was observed in 44 of the 52 isolates considered.

4.6 Correlations between SAg gene profiles and specific manifestations of GAS infection

The clinical isolates collected from STSS patients that were examined in paper II exhibited considerable diversity in terms of both their *emm*-genotypes and their SAg gene profiles. Nine different *emm*-genotypes were observed in the 30 isolates (*emm1*, *emm3*, *emm4*, *emm6*, *emm8*, *emm28*, *emm44, emm75,* and *emm85*), with 14 different SAg gene profiles between them. Six of these 14 STSS-associated SAg gene profiles were detected in the *emm1* isolates. The numbers of SAg genes in each individual STSS-associated profile ranged from two to seven, with only the *speG* gene being present in all of them. The profile with only two SAg genes consisted of the *speG* and *speJ* genes and was identified in an *emm44* isolate (Paper II).

A comparison of the 14 STSS-associated SAg gene profiles to the 18 profiles of isolates from sepsis patients without STSS showed that the *smeZ-1* and *speA* genes were significantly more common in the STSS associated profiles ($p<0.05$) (Paper II). A similarly high prevalence of the *smeZ* and *speA* genes was seen in STSS isolates in a study from the Netherlands (235). The prevalence of the *smeZ-1* and *speA* genes in the STSS profiles might simply reflect the high frequency of *emm1* isolates, which are known to frequently harbour these genes (189, 235). In our material, no specific SAg gene or combination of SAg genes was exclusively detected in the STSS associated profiles; instead similar profiles could be seen among isolates from sepsis, erysipelas, and tonsillitis patients (Paper II). In addition, the STSS profiles did not have an unusually high number of SAg genes; the mean number of SAg genes in the profiles among the STSS isolates was equal to the mean for the total GAS collection.

It could be hypothesized that all SAgs have similar abilities to trigger the systemic effects seen in STSS and that the severity of a given infection depends on the infected individual's susceptibility, which could explain both the variation in SAg gene content and the prevalence of specific SAg genes seen within the STSS associated profiles. An individual's susceptibility to SAg stimulation might depend on various predisposing factors such as their HLA-type or immune deficiencies. The uptake of a new SAg variant for which the acquired herd immunity is low into the circulating isolates would increase individual susceptibility and would be reflected in a high prevalence of these SAgs within the STSS isolates. In a study of GAS isolates collected in Japan between 1981 and 1997, Murase et al. showed that number of *emm1* isolates carrying the *speA* gene rose substantially during the end of 1980s (154). This increase in the frequency of the *speA* gene in Japanese *emm1* isolates occurred at around the same time as the outbreak of STSS seen

worldwide including Sweden in the late 1980s and early 1990s, and during which most of the isolates examined in this work were collected. In light of these results, the high frequency of *speA* and *smeZ-1* in the STSS-associated profiles may be due to a recent acquisition of these genes into the Swedish GAS population.

4.7 Neutralizing SAgs proliferative activity

The extracellular products (EP) of all of the isolates examined in this work induced proliferation in human PBMCs. No correlation between the stimulatory capacity of the EP and the *emm*-genotype of the corresponding isolate was observed. This result exemplifies that regardless of the *emm*-genotypes or the nature of the infection caused by the isolate, GAS produce mitogenic substances when cultured *in vitro* (Paper III). Several studies using clinical material have shown that the ability to neutralize SAg-induced lymphocyte proliferation protects against the development of STSS or NF during GAS infection (71, 138, 166). To compare the neutralization ability of the four disease groups (STSS, sepsis, erysipelas, and tonsillitis) examined in this work, patients were categorized as low or high responders on the basis of the ability of their acute-phase sera to neutralize the proliferation of lymphocytes induced by the EP of their infecting isolate; the cut-off value for distinguishing between high and low responders was set at 50 % neutralization. The use of EP from the infecting isolate ensured that the entire SAg repertoire expressed by respective isolate was included in the analysis. The neutralizing ability of sera from patients with STSS was found to differ significantly from that of sera from sepsis patients. Twenty of the 22 STSS patients were low responders, compared to 12 of the 20 sepsis patients ($p=0.014$). However, no significant difference in neutralization response was observed between sera from patients with bacteremia and sera from the non-bacteremic group. These results indicate that the presence of neutralizing antibodies has a profound influence on the disease outcome. However, a lack of neutralizing antibodies is not the only factor that can be used to predict STSS susceptibility. For example, of the 11 tonsillitis patients considered, only two were high responders. The outcome of a GAS induced sepsis can also be influenced by host factors such as the presence and specificity of binding epitopes on the immune cells (HLA and TCR) that effect the host cytokine response (113, 157).

4.8 Ability to develop SAg neutralizing antibodies

The capacity of the patients to develop antibodies that protect against SAg-induced mitogenic activity was determined by comparing the neutralization capacity of each patient's acute and convalescence-phase sera against EP

from their infecting GAS isolates. Of 42 initially low responders, only six produced convalescence-phase sera with an elevated capacity to neutralize EP (Paper III). Kansal et al. reported similar findings for patients who developed menstrual toxic shock syndrome after infection with toxic shock syndrome toxin-1 (TSST-1) producing *S. aureus*. In general, neither the acute- nor the convalescence-phase sera of these patients exhibited significant ability to neutralize the activity of TSST-1. A correlation was observed between the ability to neutralize the lymphocyte mitogenicity of TSST-1 and the presence of immunoglobulin isotype IgG1s in the sera (106). In our study the patient's age was found to correlate with neutralization ability. The mean age of the high responders was greater than that of the low responders, suggesting that levels of neutralizing antibodies increase with age. In a Japanese study of children with burns, who have an elevated risk of contracting toxic shock syndrome (TSS), the development of IgG antibodies that recognize the TSST-1 SAg produced was analyzed. High titers were observed in children up to six month of age, probably because of passive immunity acquired from the mother. Substantially lower titers were observed in children aged between 6 and 24 months. Titers then increased gradually with age in children older than three; 100% of all individuals between 41 and 81 years old exhibited antibody recognition of TSST-1 (187). However, it would be interesting to determine whether the acquired TSST-1 specific antibodies also protected against the mitogenic activity of the toxin.

In our study increased enzyme-linked immunosorbent assay (ELISA) titers were observed in all analyzed convalescence-phase sera showing that the mitogenic agents/SAgs had been presented to the hosts. Considering that the high ELISA titers reflects a strong Th2 response, blocking of the protective B-cell response through a vigorous Th1 response induced by the SAg does not seem to be the mechanism behind the lack of development of neutralizing antibodies. In a review of the field, Ohlin et al. have suggested that appropriate pairs of V_H and V_L genes, together with appropriate D and J segments and junctional diversity, are required for the maintenance of a sufficiently diverse human B cell repertoire that will recognize certain antigenic epitopes. The ability to respond to certain antigens or epitopes can be restricted, as shown in the cases of *Haemophius influenza* and *Cytomegalovirus*, and allelic variations in the v-gene loci have been shown to influence the diversity of the antibody response (172).

The severity and site of infection did not seem to affect the likelihood of developing EP-neutralizing antibodies, since SAg-neutralizing abilities were also observed in the convalescence sera of some tonsillitis patients who initially were low responders. In a study by Yang et al., the prevalence of antibodies capable of neutralizing individual SAgs from GAS was shown to

differ between different ethnic groups. Higher serum titers were noted in certain ethic groups; it was suggested that this was due to those groups having been more extensively exposed to streptococci in the early stages of their lives (236). Analysis of the convalescence-phase sera examined in paper III demonstrated that the host immune response to EP from the infecting isolate remained high for at least three years after the infection, indicating that the acquired neutralization capacity persists over time. Interestingly, these patients had low ELISA titers but still showed high neutralization levels. Moreover, patients whose acute-phase sera exhibited a high neutralization response retained this neutralizing ability in their convalescence-phase sera. A moderate increase in ELISA titers in the convalescences-phase sera was seen for the initially high responders; this may reflect a previously acquired B-cell memory, which would contribute to the long-term retention of neutralizing activity.

4.9 Neutralizing capacity was not general for all isolates

In paper III a correlation was observed between a lack of neutralizing ability in the acute-phase sera and the *emm*-genotype of the infecting isolate. Patients infected by *emm1* isolates were less likely to produce acute-phase sera with substantial neutralizing ability than were patients infected with isolates of other *emm*-genotypes. This indicates that the ability to acquire neutralization is not general. To determine whether the protective immune response mounted by the infecting isolate was specific for the infecting isolate or had a broader spectrum, convalescence-phase sera from patients with high neutralization capacities were tested against EP from other patients' isolates. Although most of these tests were conducted using isolates whose *emm*-genotype was the same as the patient's infecting isolate, the sera did not always exhibit neutralizing activity (Paper III). It was observed that in many cases, the year of collection affected the mitogenicity of isolates with the same *emm*-genotype. This is presumably a consequence of the presence of different mitogenic agents within the different isolates. Fig. 6 shows the SAg gene profiles for some of the isolates examined in these neutralization tests. In general, the results show that the convalescence sera were often unable to neutralize the EP mitogenicity induced by an isolate carrying one or two more SAg genes than the patient's infecting isolate (Paper II and paper III). For example, sera that neutralized the EP from an isolate carrying the *speC, speG,* and *smeZ* genes failed to neutralize the EP from an isolate carrying the *speK/L* and *ssa* genes in addition to the SAg genes of the infecting isolate (Fig. 6).

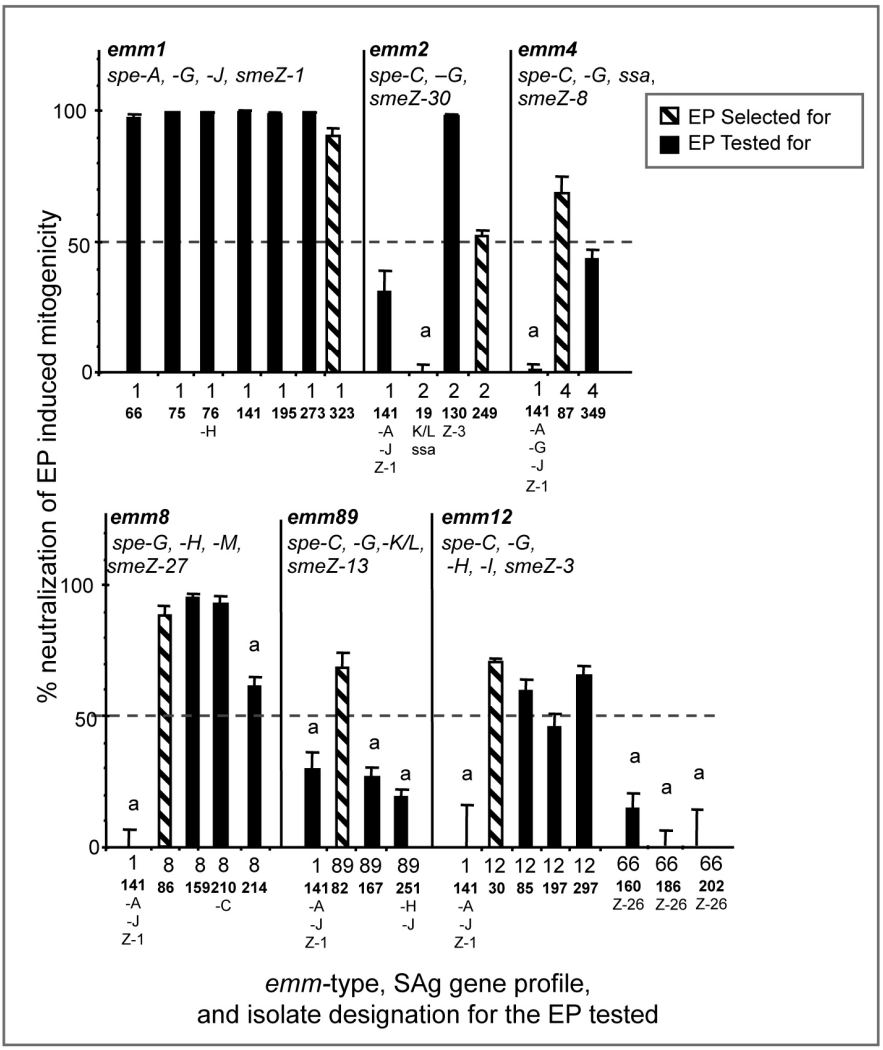

Figure 6. The capacity of individual sera to neutralize the mitogenic activity of GAS extracellular products (EP) from isolates with the indicated SAg gene profiles. The isolate *emm*-genotype, patient number, SAg gene profile, and the *smeZ* allele of the tested isolate are shown vertically on the x-axis. Only those SAg genes present in the tested isolate but not the infecting isolate are shown. The *emm*-type and SAg gene profiles of the infecting isolate and the identity of the serum tested are shown above the bars of the diagram.

4.10 Immunoglobulin or plasma treatment

The capacity of broad-spectrum anti-SAg antibodies to markedly reduce mitogenic stimulation of lymphocytes has been shown in several *in vitro* studies. The addition of IVIG significantly reduced proliferation in PBMCs, whether induced by EP from GAS isolates or by recombinant SAgs (53, 122, 161, 162, 195). Data from several clinical *in vivo* studies and case reports also indicate that immunoglobulin treatment with IVIG can be effective in the treatment of GAS-induced sepsis, STSS, or NF (37, 41, 53, 110, 119, 179). In paper III nine patients with STSS received immunoglobulin treatment with IVIG and/or plasma. For eight of these patients, acute-phase sera samples were acquired before treatment; all were low responders. Two patients received a combination of IVIG and plasma; post-treatment serum samples from these individuals exhibited neutralization levels above 50 %. The other six patients received only plasma; of these, only the two who received 15 units (originated from different individuals) or more, produced sera with SAg neutralization levels above 50 %. The larger quantity of plasma needed to acquire neutralization reflects the variable capacity of the population to neutralize SAg-induced mitogenicity. Also, the earlier reported differences in the ability of IVIG preparations to neutralize SAg activity reflect differences in neutralization ability among the population (159, 195).

CONCLUSIONS

- Recombinantly produced mitogenic factor (MF) did not induce mitogenic activity in lymphocytes as would be expected of a superantigen, but it was an active DNase. As such, and given that it is encoded by a gene that is known to be identical to that encoding DNase B in GAS, this common GAS virulence factor should be referred to as "streptococcal DNase B" (SdaB) rather than MF.

- The SAg gene content of the GAS isolates examined was found to vary both within and between different *emm*-genotypes. This implies that SAg gene profiles can be used to complement established typing methods, such as *emm*-genotyping, to further distinguish GAS isolates. Furthermore, a stable SAg gene profile was identified in a group of *emm1* isolates collected over a period of 14 years. The SAg genes within this stable profile were also the most common SAg genes identified among the other *emm1*-associated profiles, suggesting that it corresponds to a successful clone in which gain and loss of SAg genes is ongoing.

- Many of the patients examined in this thesis were found to be incapable of producing antibodies that protect against SAg-induced lymphocyte mitogenicity even though they exhibited high ELISA titers against the infecting GAS isolate in their convalescence-phase sera. This indicates that some people will always respond to infections that produce SAg antigens as though it were the first time they had encountered such species. For these individuals, a SAg-based vaccine would not confer optimal protection.

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Släkt och Vänner, Ett varmt tack till alla er för ert stöd under denna resa.
”Det finns ingen bättre spegel än en gammal vän”/Spanskt ordspråk

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