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Low Antibody Levels against Cell Wall–Attached Proteins of *Streptococcus pyogenes* Predispose for Severe Invasive Disease

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Acute-phase serum samples from 70 patients with group A streptococcal (GAS) invasive disease were analyzed for IgG antibodies against 6 recently characterized GAS virulence factors (SclA, SclB, GRAB, MtsA, EndoS, and IdeS) and SpeB. Antibody levels against the cell wall–attached GAS antigens SclA, SclB, and GRAB were significantly lower in patients with severe invasive disease (streptococcal toxic shock syndrome [STSS] and/or necrotizing fasciitis [NF]; $n = 35$), compared with levels in patients with nonsevere GAS bacteremia ($n = 35$). Among patients with severe invasive disease, significantly lower antibody levels against GRAB were found in patients with STSS ($n = 10$) than in patients with NF ($n = 17$). Antibody levels against SpeB in patients with severe bacteremia were similar to those in patients with nonsevere bacteremia, and levels in patients with STSS were similar to those in patients with NF. The data indicate that immunity to cell wall–attached proteins may play a role in the protection against severe invasive disease and that antibodies against GRAB may be of importance in the pathogenesis of STSS.

Human infections due to *Streptococcus pyogenes*, or group A streptococcus (GAS), may vary from relatively harmless infections, such as impetigo or pharyngotonsillitis, to severe invasive disease complicated by shock and multiorgan failure, such as streptococcal toxic shock syndrome (STSS) or necrotizing fasciitis (NF). During the late 1980s, there was a marked worldwide increase in the prevalence of serious invasive GAS disease, and the higher incidence has persisted [1–5]. The reason for the resurgence of invasive GAS infections is still unknown. However, it is likely due to a

combination of the emergence of more-virulent strains and the lack of protective immunity in the population.

Recently, a number of novel potential GAS virulence factors have been characterized. The cell wall–attached GRAB has been shown to bind the human proteinase inhibitor α_2 -macroglobulin [6]. By protecting bacterial surface components from proteolytic attack, GRAB might act as a virulence factor. SclA and SclB are 2 related cell wall–attached proteins that contain large regions similar to human collagen [7–11]. The NH₂-termini of both SclA and SclB are hypervariable, whereas the collagen-like regions are more conserved between proteins from different strains. Both SclA [7] and SclB [10] have been shown to increase streptococcal adherence to human cells. For the transmembrane influx of metal ions—such as Zn, Fe, and Cu—GAS is dependent on a membrane-associated protein complex. The lipoprotein component of the complex, MtsA, is needed for a sufficient uptake of metal ions [12] and for the normal growth of the bacteria in vitro [13]. EndoS and IdeS represent 2 secreted GAS enzymes that have specific effects on IgG [14–16]. EndoS removes the glycan moi-

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Table 1. Group A streptococcal antigens used in the present study.

Antigen	Size, aa	Characteristic/function	Variable structure/repeats	CWP/secreted	Protein preparation used
SclA	348–(variable)	Collagen-like, adherence?	Yes	CWP	21-mer peptide
SclB	228–(variable)	Collagen-like, adherence?	Yes	CWP	Recombinant protein fragment
GRAB	217–(variable)	α_2 -macroglobulin binding	Yes	CWP	Recombinant whole protein
MtsA	310	Metal ion transport	No	CWP	Recombinant whole protein
EndoS	959	IgG endoglycosidase	Unlikely	Secreted	Recombinant whole protein
IdeS	339	IgG degrading enzyme	Unlikely	Secreted	Recombinant whole protein, lacking enzymatic activity
SpeB	398	Cysteine proteinase	No	Secreted	Purified from culture supernatant

NOTE. CWP, cell wall protein.

ety from IgG and is thus able to alter the functionality of an opsonizing antibody. This leads to increased survival of bacteria in human blood *ex vivo*, because of impaired binding of Fc receptor [17]. IdeS is a secreted cysteine proteinase that cleaves IgG in the hinge region with a high degree of specificity. IdeS has been shown to cleave opsonizing IgG antibodies bound to streptococcal surface structures, thereby inhibiting Fc-mediated killing of GAS by phagocytic cells [16, 18].

The described novel GAS proteins all have functions that could influence virulence, and experimental data have shown that they affect bacterial adherence, multiplication, and survival. In the present study, the specific immunity to these antigens, and also to the well-characterized GAS cysteine proteinase (SpeB), was investigated in patients with invasive GAS disease.

SUBJECTS, MATERIALS, AND METHODS

Patients and serum samples. Serum samples were obtained from patients and from healthy individuals in Sweden and The Netherlands. The Swedish patients ($n = 34$) were treated for GAS bacteremia at the Clinic for Infectious Diseases, Lund University Hospital, Lund, Sweden, from 1990 to 2002. Patients from The Netherlands ($n = 36$) were part of a nationwide surveillance program conducted from 1994 to 1997 and have been described elsewhere [19]. The median and mean ages of the patients were both 50 years, (range, 0–82 years), and the male:female ratio was 34:36; all values were similar for Swedish and Dutch patients. Four individuals were <15 years old. GAS was isolated from a normally sterile site in all patients; 64 isolates were obtained from blood cultures, and 6 were obtained from a deep tissue infection. Patients were subdivided on the basis of their having either severe or nonsevere invasive infections, which was determined on the basis of the clinical course [20]. Severe infection, defined as bacteremia complicated by STSS ($n = 10$), NF ($n = 17$), or STSS with NF ($n = 8$), was present in 35 patients. Patients with nonsevere infection ($n = 35$) had no signs of hypotension, organ failure, or deep tissue

involvement. The median ages were 49 and 51 years, and the male:female ratios were 16:19 and 18:17, in the groups of patients with severe and nonsevere infection, respectively. An extensive history of disease was available from 58 of the patients. Fifteen of these were considered to have an underlying chronic disease likely to affect their immune status. These included diabetes mellitus ($n = 4$), rheumatoid arthritis ($n = 3$), ongoing malignant disease ($n = 3$), intravenous drug use ($n = 3$), and chronic use of corticosteroids ($n = 2$). Serum samples were obtained up to day 10 (mean and median, day 3.6) after onset of disease symptoms and were stored at -70°C until analysis. Additionally, control serum samples from healthy individuals were obtained from 40 Dutch and from 40 Swedish blood donors. The median and mean ages of the blood donors were 41 and 42 years, respectively (range, 19–69 years), and the male:female ratio was 42:38. The present study was approved by the Research Ethics Committee of Lund University.

Microbiology. The M serotype of Swedish streptococcal strains was determined by sequencing of *emm* gene-specific polymerase chain reaction (PCR) products (*emm* typing). For PCR amplification of *emm* genes, template DNA was prepared by boiling *S. pyogenes* bacteria in sterile water. The cell debris were removed by centrifugation, and 5 μL of the boiled lysate was used with *emm*-specific primers (primer 1, 5'-TATT(C/G)GCTTAGAAAATTAA-3'; primer 2, 5'-GCAAGTTCTTCAGCTTGTTT-3'), as described elsewhere [21]. Sequences obtained from *emm*-specific PCR products were subjected to homology searches in the *emm* sequence database at the Center for Disease Control and Prevention (available at: <http://www.cdc.gov/ncidod/biotech/strep/strepindex.html>). Isolates from The Netherlands were analyzed for M serotype, as described elsewhere [19].

Proteins and peptides. The proteins used in the present study are briefly described in table 1. The predicted mature GRAB was produced by cloning of *grab* from the GAS serotype M1 strain KTL3 [6] into the pET11d vector (Amersham-Pharmacia Biotech). The resulting plasmid was introduced into *Escherichia coli* (BL21; Amersham-Pharmacia Biotech), and trans-

formants were grown at 37°C, to an OD_{620nm} of 0.5, followed by induction with 0.5 mmol/L isopropyl β-D-thiogalactopyranoside. Bacteria were harvested by centrifugation after 3 h, were re-suspended in 20 mmol/L Tris-HCl (pH 8.0), were sonicated, and were recentrifuged at 8000 g. The lysate was subjected to ion-exchange chromatography, by use of a monoQ column (Amersham-Pharmacia Biotech) in a fast-protein liquid chromatography system (Amersham-Pharmacia Biotech). The cloning and recombinant expression of IdeS have been described elsewhere [16]. To produce an IdeS that lacks enzymatic activity, the catalytic cysteine⁹⁴ residue was replaced by a glycine by use of the Transformer Site-Directed Mutagenesis Kit (Clontech), in accordance with the manufacturer's instructions [18]. The fusion protein glutathione S-transferase (GST)-IdeS^{C94G} was purified on Glutathione-Sepharose (Amersham-Pharmacia Biotech), in accordance with standard protocols. Full-length mature EndoS and a large fragment of SclB were expressed in *E. coli* by use of GST Gene Fusion system and were purified as described elsewhere [10, 15]. MtsA was expressed and purified by the same technique but was modified to lack the N-terminal cysteine of the mature protein and, thus, also the lipid moiety [12]. SpeB was purified from the culture supernatant of an M1 *S. pyogenes* strain grown in C-medium [22] supplemented with 0.5 mmol/L dithiothreitol, as described elsewhere [23]. The purity of the proteins used in the present study was >95%, as assayed by SDS-PAGE. The SclA antigen was produced as a 21-aa long synthetic peptide (GDRGETGAQGPVGPQGEKGET). This sequence spans a region in the COOH-terminal half of the collagen-like repeats that is highly similar between SclA proteins derived from different *S. pyogenes* isolates [8]. Peptide synthesis was performed at the Biomolecular Resource Facility (Lund University, Lund, Sweden).

ELISA. Microtiter plates (Maxisorb; NUNC) were coated overnight at 4°C with GAS antigens in coating buffer (0.05 mol/L NaHCO₃ [pH 9.6]). Proteins were diluted to the following concentrations: SclA to 2 µg/mL, SclB to 0.5 µg/mL, GRAB to 0.5 µg/mL, MtsA to 0.5 µg/mL, EndoS to 0.75 µg/mL, IdeS to 0.4 µg/mL, and SpeB to 0.5 µg/mL. Plates were washed in PBST (0.05% Tween in PBS) and blocked with 2% bovine serum albumin (Sigma) in PBST (PBSTA). A fixed dilution of serum samples from patients and from healthy individuals in PBSTA was added to the wells in duplicate. The following serum dilutions were used: 1:250 for SclA, SclB, and MtsA; 1:500 for GRAB; and 1:1000 for EndoS, IdeS, and SpeB. The antigen concentrations and serum dilutions used were judged to be optimal by use of checkerboard titration. Bound antibodies were detected by incubation with a horseradish peroxidase-conjugated secondary antibody against human IgG diluted 1:3000 in PBSTA (Bio-Rad). All incubations were performed for 1 h at 37°C and followed by a washing step. Substrate solution, 0.1% (wt/vol) diammonium-2,2-azino-bis-(3-ethyl-2,3-dihy-

drobenzthiazoline)-6-sulfonate, 0.012% (vol/vol) H₂O₂ in 100 mmol/L citric acid, and 100 mmol/L NaH₂PO₄ (pH 4.5) were added, and the optical density at 415 nm was determined after 15 min. A blank without serum was included in quadruplicate on each plate, and these optical density values were subtracted from the values obtained by use of the serum samples. An ELISA index was calculated by dividing the mean optical density value for each serum sample by the mean optical density value for a standard positive serum sample run in quadruplicate on each plate. The day-to-day variation of each ELISA was 2.2%–14.2% and was expressed as a coefficient of variation on the basis of samples run on 8 different occasions.

Statistical evaluation. Comparisons between groups in which the number of observations was <40 in any group were made by use of the nonparametric Mann-Whitney *U* test. Otherwise, the 2-sample *z* test, assuming unequal variances, was used. *P* < .05 was regarded as statistically significant.

RESULTS

Patients and strain characteristics. Seventy patients with invasive GAS infection from 2 different geographical areas, The Netherlands and the southern part of Sweden, were evaluated. Thirty-five of the patients had a relatively benign disease course of GAS bacteremia without circulatory effects or organ failure, and 35 subjects had a severe infection that fulfilled the criteria for STSS and/or NF. A GAS strain was isolated from a normally sterile site from each of the patients. Of these, 57 strains were available for analysis. Strains of the M1, M28, and M3 serotypes were recovered from 17 (30%), 13 (23%), and 9 (16%) of the 57 patients, respectively. The distribution of M serotypes among patients with severe and nonsevere infection is presented in table 2. Of the 57 strains, 26 were isolated from patients with severe invasive infection. In this group, the M1 serotype represented 12 (46%) of the strains. The M28 and M3 serotypes were each isolated from 4 (15%) of the patients. The proportion of the various M serotypes was similar between the strains from

Table 2. Distribution of group A streptococcal strains of different M serotypes in patients with invasive infection.

M serotype	Severe infection	Nonsevere infection	Total
M1	12	5	17
M28	4	9	13
M3	4	5	9
Other M serotypes	6	12	18
ND	9	4	13
Total	35	35	70

NOTE. Data are no. of patients. ND, not determined (strain not available).

the Swedish and those from the Dutch patients (data not shown).

Serologic testing. IgG antibody levels against the cell wall-attached (figure 1A) and the secreted (figure 1B) streptococcal antigens were determined in serum samples from 80 healthy blood donors. All serum samples from blood donors showed detectable antibody levels against all the streptococcal antigens investigated. There were, however, considerable variations in all assays, except in the anti-SclA ELISA. No pattern with high or low antibody levels against all or several antigens could be detected on an individual basis. Furthermore, there was no difference in antibody levels against any of the antigens between the Swedish ($n = 40$) and the Dutch ($n = 40$) groups of blood donors (data not shown).

Patients with invasive GAS infection showed significantly higher acute-phase antibody levels against all antigens studied, except for EndoS, compared with blood donors (table 3). The highest antibody levels in the group of patients were observed among those with an immunocompromising condition (table

3), who showed significantly higher antibody levels against all 7 GAS antigens, compared with patients with no underlying disease.

Antibody levels against the various antigens were compared between patients with nonsevere and those with severe GAS infection. Of the 4 cell wall-attached antigens, significantly lower antibody levels ($P < .05$) were found against SclA, SclB, and GRAB in acute-phase serum samples from patients with severe infection (figure 2A). Anti-MtsA antibody levels were also lower in the group of patients with severe disease, although not significantly lower ($P = .05$). In contrast, there were no significant differences between the groups of patients when the antibodies against the secreted antigens EndoS, IdeS, and SpeB were analyzed (figure 2B).

Patients with a severe course of GAS infection developed STSS, NF, or both. Antibody levels in patients with STSS without NF ($n = 10$) and those in patients with NF without shock ($n = 17$) were compared, and, for all the investigated antigens, lower antibody levels were found in the former group. However,

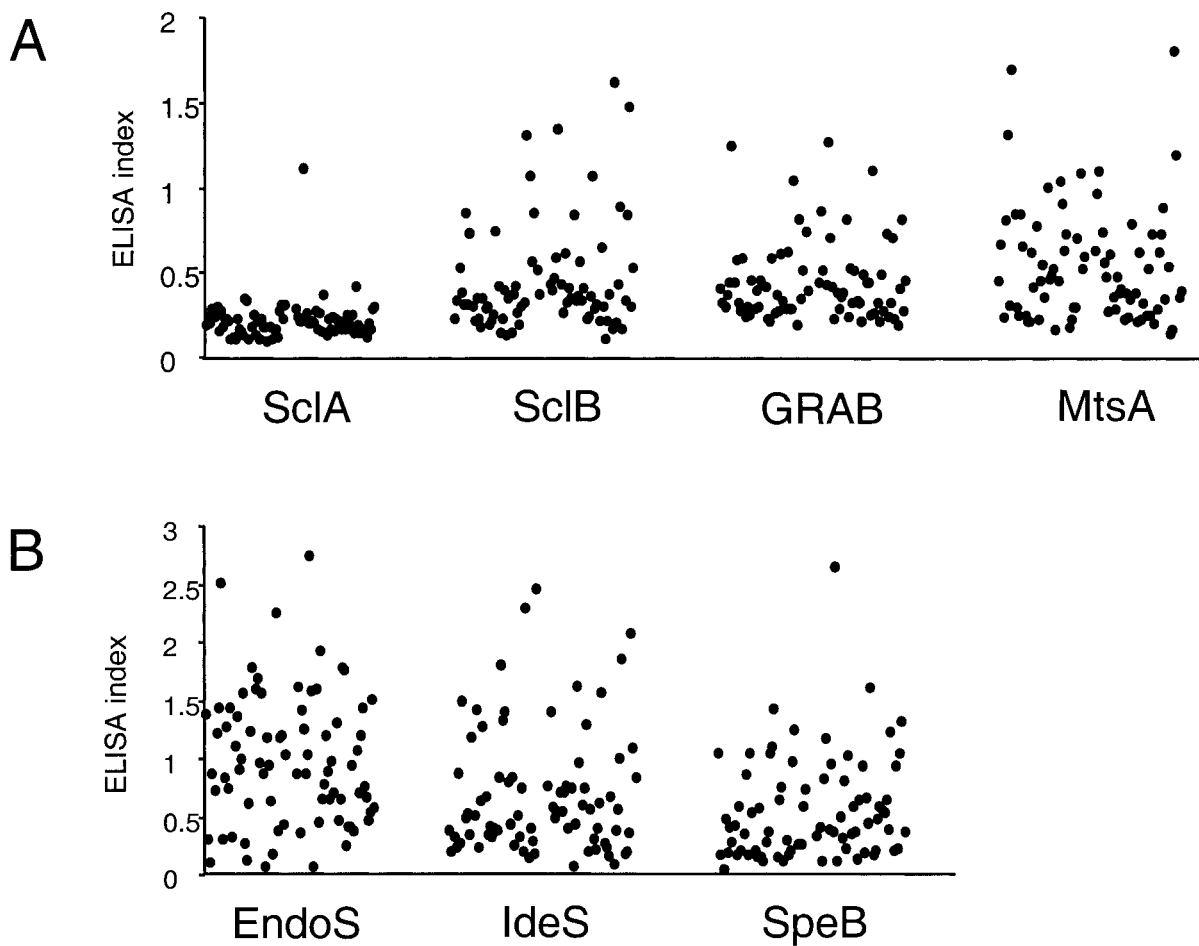


Figure 1. IgG antibody levels against group A streptococcal (GAS) antigens in serum samples from Swedish ($n = 40$) and Dutch ($n = 40$) healthy individuals. Each dot represents the ELISA index for an individual control serum sample. Levels are presented for the 4 cell wall-attached (A) and for the 3 secreted (B) GAS proteins.

Table 3. Mean ELISA indexes against group A streptococcal (GAS) antigens in blood donors and in patients with invasive GAS infection.

Antigen	Subject group		P
	Blood donors (n = 80)	Patients with invasive GAS infection (n = 70)	
SclA	0.22	0.49	<.05
SclB	0.46	0.83	<.05
GRAB	0.44	0.92	<.05
MtsA	0.51	0.88	<.05
EndoS	0.98	1.11	.23
IdeS	0.68	1.24	<.05
SpeB	0.53	1.02	<.05
Antigen	Patients with invasive GAS infection but otherwise healthy (n = 43)	Patients with invasive GAS infection with underlying chronic disease (n = 15)	P
SclA	0.39	0.69	<.05
SclB	0.70	1.05	<.05
GRAB	0.72	1.31	<.05
MtsA	0.64	1.22	<.05
EndoS	0.96	1.50	<.05
IdeS	1.01	1.65	<.05
SpeB	0.82	1.41	<.05

NOTE. Statistical calculations were made by use of the z test or the Mann-Whitney U test.

significantly lower levels were found only for anti-GRAB antibodies (figure 3A and 3B). The 8 patients who had STSS combined with soft-tissue necrosis were excluded from these statistical analyses.

DISCUSSION

A number of recently characterized GAS proteins were chosen for the present study. The genes encoding these proteins are present in all or almost all GAS isolates, but the expression of the proteins in vitro has been shown to vary. According to sequence data, the secreted enzymes EndoS, IdeS, and SpeB are conserved between GAS isolates from different M serotypes. Like several other cell wall-attached proteins, SclA, SclB, and GRAB have a more heterogeneous structure, with variable NH₂-termini and repeated regions of different size. However, large regions of SclB and GRAB are homologous between strains, justifying the selection of the entire GRAB and a large fragment of SclB as antigens. In experiments with SclA, which show a more limited identity between different GAS isolates, a 21-mer peptide constructed from the most conserved part of the molecule was used. It has previously been shown that antibodies against SclA do not cross-react with SclB, and vice versa, even

though both proteins contain large regions of collagen-like repeats [10]. MtsA, which is associated with the bacterial cell membrane through an NH₂-terminal lipid linkage, is conserved between GAS strains and shows extensive homology to related proteins in other species. Thus, detection of specific IgG against this molecule may reflect cross-reactivity with other bacterial metal permeases.

In the present study, acute-phase serum IgG antibody levels against the 6 newly described GAS virulence factors were investigated. In addition, SpeB was included, since it is considered to be an important virulence factor. This proteinase is expressed in all GAS strains, and serologic data were available from other studies [19, 24–26]. Detectable antibody levels against all GAS antigens investigated were found in all serum samples from healthy individuals (figure 1). This probably reflects the common exposure to GAS in the general population, and it supports a ubiquitous presence of these antigens in different GAS isolates. Thus, when comparing the serum samples from healthy Swedish individuals with those from healthy Dutch individuals, there were no differences in antibody levels against the 7 streptococcal antigens studied. However, the in vivo expression of specific streptococcal antigens and their immunogenicity is probably variable, since, among individual blood donors, there was a significant heterogeneity in antibody levels against the different antigens. A similar individual variation in antibody levels was seen among 30 adult patients with acute-phase GAS pharyngotonsillitis, but, in general, there was no difference in specific antibody levels between blood donors and patients with pharyngotonsillitis (P. Åkesson and B. Christensson, unpublished data). In all assays, the 4 children <15 years old showed antibody levels that were similar to those showed by the adult patients and were therefore included in the present study. The distribution of different M serotype strains among the blood culture isolates from the Swedish and the Dutch patients was also similar. Taken together, serum samples and bacterial strains from patients from Sweden and those from patients from The Netherlands were comparable, and they were, therefore, not separated in the present study. Moreover, the higher prevalence of the M1 serotype observed in patients with complicated GAS bacteremia is in accordance with data from several other studies [27, 28].

When acute-phase antibody levels in patients with severe disease (STSS and/or NF) were compared with those in patients with an uncomplicated course of GAS bacteremia, lower antibody levels against cell wall-attached antigens were found in the group of patients with severe disease (figure 2). ELISA indexes for SclA, SclB, and GRAB were significantly lower ($P < .05$) among the patients with severe disease, although there was no significant difference for MtsA ($P = .050$). Previous studies have shown that a low antibody response against the cell wall-attached M1 protein was seen in patients with GAS bacteremia, compared with that seen in healthy individuals

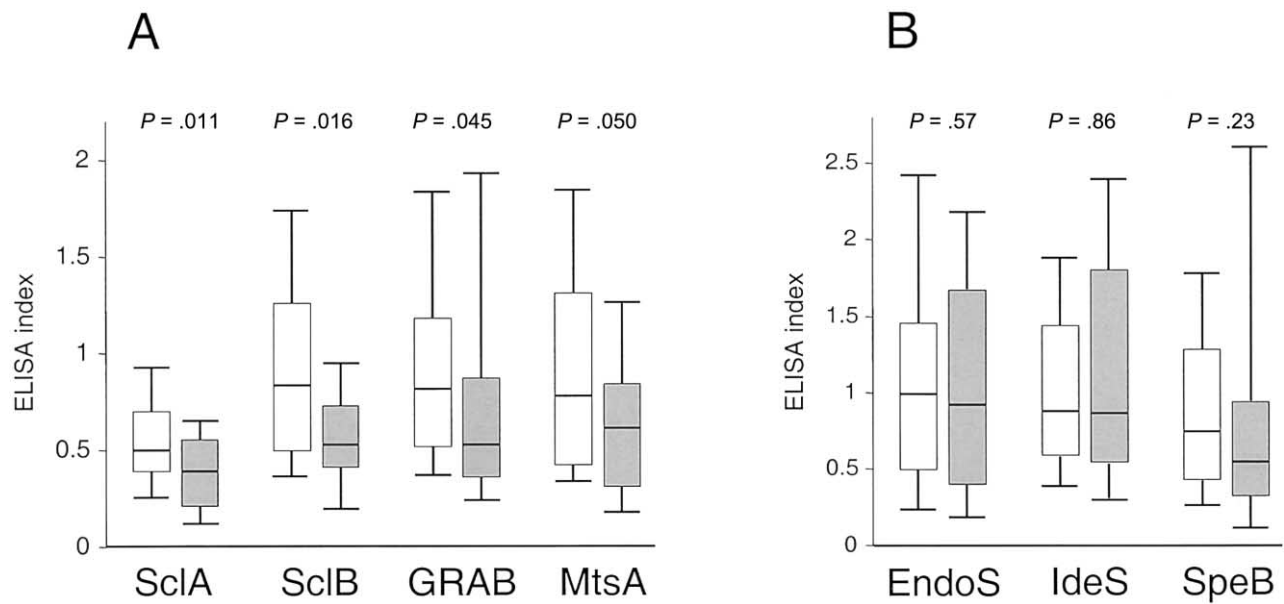


Figure 2. Acute-phase IgG antibody levels in patients with nonsevere and severe invasive group A streptococcal (GAS) disease. Levels against cell wall-attached (A) and secreted (B) GAS proteins are expressed as ELISA indexes. White boxes represent patients with nonsevere GAS bacteremia ($n = 35$), and gray boxes represent patients with severe invasive infection ($n = 35$). The median value and the 25th and 75th percentiles are marked by boxes. Horizontal lines outside the boxes represent the 10th and 90th percentiles. Statistical calculations of P values were made by the Mann-Whitney U test.

[24], and in patients with fatal sepsis, compared with that seen in patients with tonsillitis [25]. The low antibody response against SclA, SclB, and GRAB suggests that immunity against GAS cell wall components is of importance for the outcome of invasive GAS disease. On the other hand, antibody levels against the secreted antigens EndoS, IdeS, and SpeB did not differ between the 2 groups of patients. The data for SpeB are in contrast to those from 2 previous studies [25, 26] that demonstrated that low antibody levels against SpeB is a risk factor for developing complicated GAS bacteremia, including STSS. However, data from 2 other studies [19, 24] showed similar anti-SpeB levels in patients with severe and in those with nonsevere bacteremia, respectively, which is in accordance with the results presented here. In the present study, there are no data on the absolute concentration of specific serum antibodies or on the exact level of bacteremia. It could be argued that the low level of antibodies against cell wall-attached proteins found in patients with severe disease is due to absorption of antibodies by a high number of circulating streptococcal organisms. However, data from previous serological studies on patients with *Staphylococcus aureus* endocarditis, in which there is a sustained high level of bacteremia, even during appropriate antibiotic therapy, do not support this assumption. Thus, higher levels of IgG antibodies against *S. aureus* capsular polysaccharide, a surface antigen, and *S. aureus* lipase, a secreted enzyme, were found in patients with endocarditis than in patients with uncomplicated low levels of *S. aureus* bacteremia [29, 30].

In the previous definition of STSS, a soft-tissue necrosis including NF could be 1 sign required to fulfil the criteria for a definite case of STSS [20]. However, in the same consensus document, other invasive GAS disease could include focal infections, such as NF, with or without bacteremia but without shock. Since these patients with NF appear clinically different, it could be assumed that other pathogenic factors are involved. Thus, we separately investigated patients with STSS without NF and patients with NF without shock. When antibody levels were measured in these 2 groups, a significantly lower level against GRAB was found in patients with STSS without NF. These data indicate that low IgG antibody levels against GRAB might be a risk factor for developing STSS in patients with GAS bacteremia. The function of GRAB is intimately related to regulation of proteolysis and its control at the bacterial surface [31]. The action of GRAB in vivo could possibly lead to an alteration of proteolytic mechanisms involving the complement, the contact, and the coagulation systems, thus affecting the severity of the disease.

Contrary to most previous studies [19, 24–26], the present study found that antibody levels against all antigens investigated were significantly higher in the acute-phase serum samples from patients with invasive GAS disease than in those from the healthy blood donors (table 3). A similar result was seen in an earlier study, in which the mean IgG level against SpeB among patients with GAS bacteremia was higher than that among patients with tonsillitis [25]. A subgroup analysis of the pres-

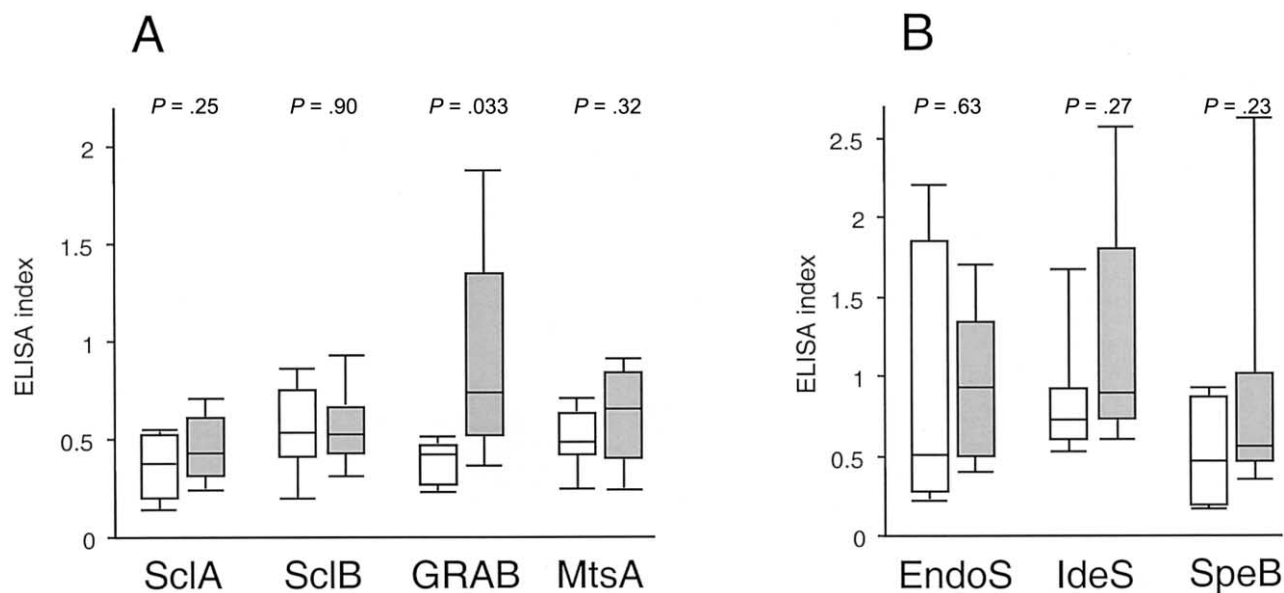


Figure 3. Acute-phase IgG antibody levels in patients with severe invasive group A streptococcal (GAS) disease. White boxes represent patients with streptococcal toxic shock syndrome without necrotizing fasciitis (NF) ($n = 10$), and gray boxes represent patients with NF without shock. Levels against cell wall-attached (A) and secreted (B) GAS proteins are expressed as ELISA indexes. The median value and the 25th and 75th percentiles are marked by boxes. Horizontal lines outside the boxes represent the 10th and 90th percentiles. Statistical calculations of P values were made by use of the Mann-Whitney U test.

ent data showed that patients with an underlying immunocompromising condition had higher antibody levels against all the studied GAS antigens, compared with patients who were healthy before the streptococcal infection (table 3). Hypothetically, this could be explained by the more prevalent occurrence of streptococcal skin and mucous membrane infections seen, for example, among diabetic patients, drug addicts, and patients with rheumatoid arthritis, although no such data on medical history were available in the present data. It might also be argued that a boosted IgG immune response could already have taken place, up to 10 days after the onset of infection. However, this possibility was not confirmed when serologic data were plotted according to time of serum sampling in relation to onset of infection (data not shown), and mean and median serum sampling were obtained only 3.6 days after onset of infection. Taken together, the results indicate that patients who develop invasive GAS disease usually have been previously exposed to GAS, as reflected by a preexisting immune response. This illustrates that not only the immune status, but also other host factors, must play a role in determining susceptibility to invasive GAS disease. For instance, a recent study has shown that STSS and NF are associated with specific HLA class II haplotypes [32].

In conclusion, significantly lower preexisting IgG antibody levels against 3 newly described cell wall-attached GAS antigens (SclA, SclB, and GRAB) were detected in patients with severe invasive GAS infection than in those with nonsevere invasive GAS infection. Additionally, patients who developed STSS with-

out NF showed significantly lower antibody levels against GRAB, compared with patients with NF without shock. These findings underline the pathogenic significance of interactions between surface proteins of GAS and molecules of the human host.

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