Human Immune Response to Streptococcal Inhibitor of Complement, a Serotype M1 Group A *Streptococcus* Extracellular Protein Involved in Epidemics

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Streptococcal inhibitor of complement (Sic) is a highly polymorphic extracellular protein made by serotype M1 group A *Streptococcus* strains that contributes to bacterial persistence in the mammalian upper respiratory tract. New variants of the Sic protein arise very rapidly by positive selection in human populations during M1 epidemics. The human antibody response to Sic was analyzed. Of 636 persons living in diverse localities, 43% had anti-Sic serum antibodies, but only 16.4% had anti-M1 protein serum antibody. Anti-Sic antibody was also present in nasal wash specimens in high frequency. Linear B cell epitope mapping showed that serum antibodies recognized epitopes located in structurally variable regions of Sic and the amino terminal hypervariable region of the M1 protein. Phage display analyses confirmed that the polymorphic regions of Sic are primary targets of host antibodies. These results support the hypothesis that selection of Sic variants occurs on mucosal surfaces by a mechanism that involves acquired host antibody.

Group A *Streptococcus* (GAS) is a human-adapted pathogen that causes a variety of diseases, including pharyngitis (strep throat), cellulitis, impetigo, necrotizing fascitis, and streptococcal toxic shock syndrome. Streptococcal inhibitor of complement (Sic) is a GAS extracellular protein that was initially described on the basis of its ability to inhibit the cytolytic activity of the complement membrane attack complex in vitro [1]. The exact mechanism by which Sic interacts with the membrane attack complex to inhibit lysis is unknown, and it is not clear what role this activity plays in vivo. The Sic protein is made predominantly by M1 organisms, 1 of >100 distinct serotypes of this cell-surface protein. M1 GAS strains are the most common serotype cultured from human invasive disease episodes in most case series. M1 GAS strains have a well-documented propensity to exhibit pronounced shifts in disease frequency and severity, resulting in epidemics of human infections that generally last 2–3 years (reviewed in [2]). The molecular mechanisms contributing to the abundance of M1 strains in invasive infections and their epidemic behavior are poorly understood.

Virtually all serotype M1 GAS strains are closely related genetically as shown by many molecular methods, including comparative sequencing of 15 chromosomal genes [2–7]. In general, it is uncommon to identify gene sequence polymorphisms (allelic variation) among natural populations of M1 isolates, even organisms recovered decades apart [2]. However, sequencing of the *sic* gene from 1132 serotype M1 GAS isolates recovered from global sources and responsible for a range of diseases showed a remarkably high level of allelic diversity, with 220 distinct variants found to code for 215 Sic protein variants [8]. Several other analyses of *sic* variation together have iden-
tified >300 distinct sic alleles in natural populations of M1 strains [8–12]. The level of sic variation also far exceeds that found in the region of the emm1 gene that encodes the hyper-variable amino terminal portion of the antiphagocytotic M1 protein [4, 8, 13], a key GAS virulence factor presumed to be under strong immune selection. Amino acid replacements in the amino terminus of M1 protein can alter recognition by opsonic antibodies, suggesting that these structural variants arise after host antibody selection [13]. Importantly, virtually all sic gene polymorphisms result in structural changes in the Sic protein, and most amino acid replacements are radical changes (i.e., polar-nonpolar, nonpolar-polar, or those resulting in charge changes), together indicating that very strong positive (Darwinian) selection acts on sic.

In addition to the high level of sic diversity identified in M1 GAS isolates cultured from persons living in many countries, Hoe et al. [8] recently discovered that Sic variants are rapidly selected during distinct epidemics of invasive disease caused by serotype M1 organisms previously thought to be mono- or pancreatic. Identification of many sic gene variants in a sample of M1 pharyngitis isolates recovered during epidemics suggested that Sic variants were selected on the human mucosal surface rather than after invasion of a normally sterile host site such as blood or deep tissues [8]. The concept of selection on the mucosal surface was supported by the identification of Sic variants among GAS isolates cultured over time from the throats of persistently colonized mice and among epidemiologically linked human patients [8]. More recently, Lukomski et al. [14] reported that an isogenic mutant M1 strain that does not produce Sic protein was significantly impaired in its ability to persist in the upper respiratory tract of the mammalian host during early host-pathogen interaction. Together the data suggest that the host mucosa is a site of selection of Sic variants.

Despite these advances, many issues critical to understanding the molecular basis of epidemics of M1 GAS strains and host-pathogen interactions have not been addressed. Among these is the important need to identify host factors bearing on rapid selection of Sic variants. Recovery of Sic variants at high frequency from the throats of mice after 5–6 weeks of persistent GAS colonization, but not earlier, suggested that acquired host immunity was involved in selection [8]. However, there are no data bearing on the human antibody response to Sic. To gain further understanding of Sic-host interactions and M1 epidemics, we analyzed the human immune response to Sic and the amino terminus of M1 protein in diverse populations.

Methods

Sic purification. The Sic1.01 protein variant was purified from the culture supernatant of MGAS 5005 grown in Todd-Hewitt broth supplemented with 0.2% yeast extract, as described elsewhere [8]. The sic1.01 allele is the most common allele identified in M1 strains from global sources. The sic1.01 allele has been identified in 27% of 1132 isolates sequenced [8]. The protein was >98% pure, as assessed by SDS-PAGE and Coomassie blue staining.

Serum and nasal wash specimens. The 636 serum specimens from healthy volunteers analyzed in this study are described in Table 1. Two sets of serum samples were collected in Houston. Serum samples were obtained from 119 healthy young adults from the Houston metropolitan area as part of a pneumococcal carriage study. Nasal wash specimens obtained from these subjects were also analyzed. Eighty-nine serum samples were obtained from non-case family members enrolled in a study examining the human genetics of susceptibility to tuberculosis in Houston. Additional serum samples were collected from persons living in Tennessee, New York, Finland, and Ontario, Canada. Serum samples were obtained from 98 healthy children living in the Nashville area who were participants in an influenza vaccine trial. Ninety-six serum samples were obtained from healthy children living in the Rochester, New York, area who were enrolled in vaccine trials conducted through the Elmwood Pediatric Practice. Serum samples obtained from 122 persons living in Finland were collected at 15 health centers throughout the country as part of diverse seroepidemiologic studies conducted by the National Public Health Institute in Helsinki. The subjects were healthy (i.e., no acute infections or antimicrobial therapy) at the time of sampling. Serum samples obtained from 112 persons living in Ontario were collected as part of routine blood donations. None of the study subjects had a known recent history of exposure to GAS. The date of the last known pharyngitis episode for these subjects is unknown.

Sequential human serum samples. Sequential serum samples obtained from a cohort of 61 adults were available in an archive at Rocky Mountain Laboratories. These serum samples were obtained between 1981 and 1999 from persons with no known history of invasive GAS disease or recent GAS pharyngitis. The interval between paired serum samples was 3–18 years (mean, 13 years). The date of the last known pharyngitis episode for these subjects is unknown.

Measurement of anti-Sic IgG in human serum. Microtiter plate wells (Immulon 1; Dynatech Laboratories, Chantilly, VA) were coated with 100 μL of a solution of purified sic1.01 (10 μg/mL in carbonate buffer [pH 9.6]). The Sic1.01 protein used in this assay was >98% pure, as assessed by SDS-PAGE and Coomassie blue staining [8]. The plates were incubated for 1 h at room temperature and washed 3 times with 100 μL of PBS containing 0.1% Tween 20 (TPBS) by automated plate washer (Tri Continent MultiWash PLUS, Molecular Devices, Sunnyvale, CA). The wells were blocked for 30 min at 37°C with Super Block (ScyTek Laboratories, Logan, UT). Fourfold dilutions of 20 μL serum were added to wells along with 100 μL of a solution of puriﬁed protein, 5% fetal calf serum, and 0.1% Tween 20 (100 μL/more dilution). Microtiter plate wells were incubated at 37°C for 1 h with rocking. Wells were washed 3 times with 200 μL TPBS and 100 μL p-nitrophenyl phosphate (Bio-Rad Laboratories, Hercules, CA). Optical density at 405 nm (OD405) was determined in an automated spectrophotometer (SpectraMAX 250, Molecular Devices, Sunnyvale, CA). OD405 values were analyzed by linear regression, and an arithmetic mean IgG serum antibody titer was calculated. The mean antibody titer was used as a measure of anti-Sic IgG in the serum samples. The sera were run in triplicate and contained a positive control serum and a sample of normal serum.

Table 1. Human serum samples (n = 636) analyzed.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Age range, years</th>
<th>% Females/ % males</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texasb</td>
<td>26.1–43.7</td>
<td>56.3/43.7</td>
<td>119</td>
</tr>
<tr>
<td>New York</td>
<td>0.2–22</td>
<td>Not available</td>
<td>96</td>
</tr>
<tr>
<td>Tennessee</td>
<td>4–10</td>
<td>42.9/57.1</td>
<td>98</td>
</tr>
<tr>
<td>Finland</td>
<td>18–58</td>
<td>50.8/49.2</td>
<td>122</td>
</tr>
<tr>
<td>Ontario, Canada</td>
<td>2–93</td>
<td>46.4/53.6</td>
<td>112</td>
</tr>
</tbody>
</table>

a Serum samples were obtained from T.R.C., Baylor College of Medicine, Houston.
b Plasma samples from noncase subjects were obtained through the Baylor Pediatric Tuberculosis Project, Houston.
UT) and washed 3 times in TPBS. To determine the levels of anti–Sic IgG in the 636 human serum samples, plates were incubated for 2 h at 37°C with 100 μL of patient serum samples diluted 1:1000 in PBS. To determine the levels of anti–Sic IgG in serial serum samples from 16 persons, plates were incubated as described above with 100 μL of serial 2-fold dilutions of serum starting from 1:100. Human serum samples that did not react with Sic1.01 were used as negative controls. The plate blank consisted of 100 μL of PBS. The plates were washed 3 times with TPBS and incubated for 2 h at 37°C with 100 μL of horseradish peroxidase-conjugated goat anti–human IgG (heavy and light chains; Bio-Rad Laboratories, Hercules, CA) diluted 1:2000 in TPBS. The plates were washed 3 times with TPBS and incubated with ABTS (Boehringer Mannheim Biochemicals, Indianapolis) as the development agent for 15 min at room temperature in the dark. The absorbance was measured at 405 nm with a Spectramax PLUS instrument (Molecular Devices). The 636 serum samples were considered to have specific Sic antibody if the absorbance values were greater than the average of the negative controls (i.e., human serum samples that did not react with Sic1.01) plus 3SD. The absorbance was measured automatically at 1-min intervals for 20 min at 25°C with intermittent shaking with a Spectramax PLUS instrument (Molecular Devices). Results were expressed as Vmax, which is the maximum slope of the kinetic display of optical density (OD) versus time, expressed in milli-OD units per minute (mOD/min).

Measurement of anti–M1 immunoglobulin in human serum samples. Microtiter plate wells (Nunc-Immuno Modules Maxisorp F16; Nalge Nunc International, Rochester, NY) were coated with 100 μL of a solution of a recombinant amino terminal peptide of the M1.0 protein (5 μg/mL in carbonate buffer [pH 9.6]) and incubated for 16 h at room temperature. The peptide contains 2 tandem repeats of the N-terminal 50 amino acids (aa) of the mature M1.0 protein [15]. Plates were washed 8 times with 100 μL of normal saline containing 0.15% Tween 20 (saline-Tween) and incubated for 2 h at 37°C with 100 μL of human serum diluted 1:400 in PBS containing 0.05% Tween 20 (PBS-Tween). To determine the levels of anti–M1 immunoglobulin in serial serum samples from 2 subjects, plates were incubated as described above with 100 μL of serial 2-fold dilutions of serum starting at 1:100. Human serum samples that did not react with the M1.0 peptide were used as negative controls. Rabbit serum samples against recombinant M1.0 protein were used as a positive control. The plate blank consisted of 100 μL of PBS. The plates were washed 8 times with saline-Tween and incubated for 2 h at 37°C with 150 μL of horseradish peroxidase-conjugated goat anti–human immunoglobulin (ICN Biomedicals, Aurora, OH) diluted 1:2000 in PBS-Tween or 100 μL of horseradish peroxidase-conjugated goat anti–rabbit immunoglobulin (ICN Biomedicals) diluted 1:2000 in PBS-Tween. The plates were washed 8 times in saline-Tween and incubated with 150 μL of 5 amino-salicylic acid (Aldrich Chemical, Milwaukee) for 15 min. The absorbance was measured at 450 nm by microplate reader (MR 600; Dynatech Laboratories). Samples were considered to have specific M1.0 antibody if the absorbance values were greater than the average of the negative controls (i.e., human serum samples that did not react with the M1.0 peptide) plus 3SD.

Human nasal wash kinetic ELISA. We used an ELISA to determine the levels of total IgA or IgG and the relative concentrations of Sic–specific IgA or IgG in human nasal wash samples. Microtiter plate wells (Immulon 1) were coated with 100 μL of a solution of goat anti–human IgA or goat anti–human IgG (10 μg/μL in carbonate buffer [pH 9.6]; Chemicon International, Temecula, CA) or 100 μL of a solution of purified Sic1.01 (10 μg/mL in carbonate buffer [pH 9.6]) and incubated for 16 h at room temperature. Wells were washed 3 times with TPBS and blocked for 30 min at 37°C with PBS containing 0.1% bovine serum albumin (BSA). After 3 washes with TPBS, 100 μL of nasal wash, serially diluted 5-fold from 1:2 in TPBS containing 0.1 M EDTA was added to antibody-coated wells (for determination of total immunoglobulin) and Sic-coated wells (for determination of Sic-specific immunoglobulin). Standards consisted of 100 μL of purified human secretory IgA or purified human IgG (ICN Biomedicals) serially diluted 2-fold from 1 μg/mL and added to antibody-coated wells. The plate blank consisted of 100 μL of PBS. After a 2-h incubation at room temperature, the plates were washed 3 times with TPBS and incubated for 2 h with horseradish peroxidase-conjugated goat anti–human IgA (ICN Biomedicals) diluted 1:64,000 in TPBS or horseradish peroxidase-conjugated goat anti–human IgG (Chemicon International) diluted 1:100,000 in TPBS. After 3 washes with TPBS and 1 wash with PBS, 100 μL of ABTS was added to each well and the absorbance at 405 nm was measured automatically at 1-min intervals for 20 min at 25°C with intermittent shaking with a Spectramax PLUS instrument (Molecular Devices). Results were expressed as Vmax, which is the maximum slope of the kinetic display of optical density (OD) versus time, expressed in milli-OD units per minute (mOD/min).

Linear B cell epitope mapping. Ninety 15-mer synthetic peptides spanning the entire mature Sic1.01 protein variant (281 aa) were purchased from Chiron Technologies (San Diego). Each sequential peptide overlapped the preceding peptide by 12 aa, except for the last 2 peptides, which had an overlap of 13 aa residues. Fifty-nine 15-mer synthetic peptides spanning the amino terminal variable region of the M1.0 protein (aa 27–119; GenBank accession no. X07933) [4, 11, 13] were purchased from Chiron Technologies. Each sequential M1.0-specific peptide spanning aa 27–89 overlapped the preceding peptide by 14 aa, and each sequential M1.0-specific peptide spanning aa 89–119 overlapped the preceding peptide by 12 aa. Sic1.01 and M1.0 peptides were covalently linked to biotin at the amino terminus by a serine-glycine-serine-glycine spacer. Each peptide was received as a dried powder and reconstituted with 200 μL of 99.9% dimethyl sulfoxide (Sigma, St. Louis).

Streptavidin-coated plates (Chiron Technologies) were washed 3 times with TPBS by Tri Continent MultiWash PLUS automated plate washer (Molecular Devices). The plates were blocked for 16 h at room temperature with Super Block (ScyTek Laboratories) and washed 3 times with TPBS. The peptides were diluted 1:1000 from an 8.5 mg/mL stock with 1× PBS containing 0.1% azide, added to each well (100 μL/well), and incubated for 1 h at room temperature. Wells were washed 3 times with TPBS and blocked for 30 min at 37°C with PBS containing 0.1% bovine serum albumin (BSA). After 3 washes with TPBS, 100 μL of nasal wash, serially diluted 5-fold from 1:2 in TPBS containing 0.1 M EDTA was added to antibody-coated wells (for determination of total immunoglobulin) and Sic-coated wells (for determination of Sic-specific immunoglobulin). Standards consisted of 100 μL of purified human secretory IgA or purified human IgG (ICN Biomedicals) serially diluted 2-fold from 1 μg/mL and added to antibody-coated wells. The plate blank consisted of 100 μL of PBS. After a 2-h incubation at room temperature, the plates were washed 3 times with TPBS and incubated for 2 h with horseradish peroxidase-conjugated goat anti–human IgA (ICN Biomedicals) diluted 1:64,000 in TPBS or horseradish peroxidase-conjugated goat anti–human IgG (Chemicon International) diluted 1:100,000 in TPBS. After 3 washes with TPBS and 1 wash with PBS, 100 μL of ABTS was added to each well and the absorbance at 405 nm was measured automatically at 1-min intervals for 20 min at 25°C with intermittent shaking with a Spectramax PLUS instrument (Molecular Devices). Results were expressed as Vmax, which is the maximum slope of the kinetic display of optical density (OD) versus time, expressed in milli-OD units per minute (mOD/min). We used a curve-fitting program (SOFTmax PRO version 2.6.1; Molecular Devices) to construct a reference curve for each ELISA plate from the Vmax values of the purified standards. Total and Sic-specific antibody concentrations were interpolated from the reference curve. Samples with total IgA and IgG concentrations <1 μg/mL and 0.6 μg/mL, respectively, were omitted from the analysis. Samples with specific IgA concentrations >60 ng/mL were included in the analysis and assigned a value of 30 ng/mL. Samples with specific IgG concentrations <25 ng/mL were included in the analysis and assigned a value of 12.5 ng/mL. Sic-specific antibody results were expressed as a percentage of total immunoglobulin, to account for variation in total immunoglobulin content in the samples.

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temperature. The plates were washed 3 times with TPBS and 100 µL of human serum samples diluted 1:200 in PBS was added to each well and incubated for 2 h at room temperature. The plates were washed 4 times with TPBS and 100 µL of a 1:2000 dilution of horseradish peroxidase–conjugated goat anti–human IgG (heavy and light chains; Bio-Rad Laboratories) was added. The plates were incubated for 1 h at room temperature, washed 4 times with TPBS and twice with PBS, and incubated for 20 min at room temperature in the dark with 100 µL of ABTS. The absorbance at 405 nm was measured with a Spectramax PLUS instrument (Molecular Devices). A ratio cutoff was used to determine positive immunoreactivity for each peptide [16]. For each serum sample, the lowest 25% of the absorbance values (when taking all 90 peptides into consideration) were averaged, and the absorbance value for each of the 90 peptides was divided by the average. Ratios ≥3 were scored as positive.

**Construction of Sic fragment library in pTP179 phage display vector.** The Sic phage display library was constructed by amplifying DNA encoding the sic1.01 allele from strain MGAS 5005. The gene region amplified encodes the mature portion of the Sic protein without the secretion signal sequence. The primers used were Sic-1 5′-GGCGGGGTCTGACAGAAGCTATACATCACGCAATTTTGAC and Sic-2 5′-GGCGGGTGCTAGACCGTTCGTATGGTGATATGGTGAC. To create a set of deletion fragments for library construction, ~5 µg of the 872-bp polymerase chain reaction (PCR) product was digested with BsaI nuclease (New England Biolabs, Beverly, MA) at 30°C for a time sufficient to generate fragments ranging from 870 bp to 100 bp as assessed by agarose gel electrophoresis. The digests were made at 30°C in 600 mM NaCl, 12 mM CaCl₂, 12 mM MgCl₂, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA. The reaction was stopped by the addition of EDTA to a final concentration of 25 mM. The resulting fragments were purified with a Qiaquick column (Qiagen, Valencia, CA). The ends of the BsaI-generated deletions were made blunt with 4 U of T4 DNA polymerase (New England Biolabs) in 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, 100 mM dNTPs, and 50 µg/mL BSA. The reaction was incubated at 12°C for 20 min, and the DNA fragments were purified with a Qiaquick column and resuspended in 40 µL of H₂O.

The phage display vector used for these experiments (pTP179) permits the expression of open-reading frames fused to the gene III protein of filamentous phage M13 [17]. The expression of the fusion protein is under the control of the arabinose BAD promoter [18]. In total, 5 µg of pTP179 plasmid was digested with SaI and XbaI, and the resulting product was end filled with Klenow DNA polymerase and 0.2 mM dNTPs. The digested plasmid was purified by Qiaquick column after agarose gel electrophoresis. The fragmented sic DNA was then ligated to the pTP179 vector DNA and used to transform *Escherichia coli* TG1 by electroporation. A total of 68,500 transformants were pooled to create the library.

**Phage preparation.** The initial preparation of phages from the library was done with the pooled colonies obtained from the transformation of the ligation reaction. The culture was diluted to OD₆₅₀ 0.6 in 25 mL of 2YT medium supplemented with 12.5 µg/mL chloramphenicol. VCS M13 helper phage were added at an MOI of 20, and arabinose was added to 0.2%. After overnight growth at 37°C, the *E. coli* TG1 cells were removed by centrifugation, and the phages were precipitated from the supernatant with 1/5 volume of 20% polyethylene glycol, 2.5 M NaCl. The phages were recovered by centrifugation and resuspended in 1/100 of the original culture volume of STE (0.1 M NaCl, 10 mM Tris-Cl [pH 8.0], and 1 mM EDTA [pH 8.0]). The phage titer was determined with serial dilutions of 100 µL of phage and 200 µL of *E. coli* TG1 cells. Aliquots of 150 µL were plated on LB agar supplemented with 12.5 µg/mL chloramphenicol. After overnight growth at 37°C, the number of colonies was determined, and the titer was calculated.

**Sic phage panning.** Panning was done by coating the wells of a 96-well microtiter plate with 200 µL of a 10 µg/mL solution of monoclonal or polyclonal serum samples or BSA in 0.05 M Na₂CO₃ (pH 9.6) overnight at 4°C. The wells were then treated with 200 µL of Super Block (Pierce, Rockford, IL) for 1 h, to minimize nonspecific binding. The wells were washed 4 times with 200 µL of PBS wash buffer [19] containing 1 mg/mL BSA and 0.5 g/L Tween 20. In total, 10¹¹ phages were added to each well in a 200-µL volume and allowed to bind for 2 h at room temperature. The wells were then washed 10 times with 200 µL of wash buffer, and bound phages were eluted from the wells by incubation with 200 µL of elution buffer (0.1 M glycine [pH 2.2], 1 mg/mL BSA, 0.5 g/L Tween 20, and 0.5 M KC1) for 30 min. The elution mixture was neutralized with 25 µL of 1 M Tris-Cl, pH 8.0. The phage titer of the elution mixture was determined as described above. The eluted phages were amplified by adding 150 µL of the neutralized elution to 1 mL of *E. coli* TG1 cells. After a 30-min incubation at room temperature, 25 mL of 2YT medium was added along with 10³ VCS M13 helper phage (Stratagene, La Jolla, CA). The phages were precipitated as described above after overnight incubation at 37°C with shaking. The phage titer was determined, and panning was repeated for a total of 3 rounds. After the third round of panning, the eluted phages were used to infect *E. coli* TG1, and several of the resulting colonies were randomly selected for DNA sequence analysis. The sic gene inserts were amplified by PCR with primers complementary to regions in pTP179 located on either side of the SaI and XbaI sites used for cloning. The same primers were used to determine the DNA sequence of the sic inserts with the PCR products as template.

**Statistical analysis.** We used Pearson’s correlation coefficient to assess the relationship between anti–Sic and anti–M1 serum IgG levels. The relationships between nasal wash immunoglobulin and serum IgG levels were analyzed by linear regression methods. Statistical differences between Sic-specific nasal wash immunoglobulin levels in anti-Sic serum IgG-positive and -negative groups were evaluated by Student’s t test. Statistical calculations were performed with SAS software (SAS Institute, Cary, NC).

**Mouse monoclonal antibody (MAb) to Sic1.01.** A mouse MAb was generated against purified Sic1.01 by standard procedures (Covance Research Products, Richmond, CA). Linear epitope mapping of this antibody (4H7-C5-H4) identified peaks of reactivity to Sic-specific peptide 7 (DDWSGDDPWEDDWSG) and peptide 20 (SDEKWWPDWEDD), suggesting the minimum epitope recognized by this antibody is DWPEDD.

**Results**

**Presence of Sic and M1 antibodies in human serum samples.** The significant excess of nonsynonymous nucleotide changes
Presence of anti-streptococcal inhibitor of complement (Sic) and anti-M1 IgG in 636 human serum samples.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Anti-Sic IgG</th>
<th>Anti-M1 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texas(^a)</td>
<td>60/119 (50)</td>
<td>18/119 (15)</td>
</tr>
<tr>
<td>Texas(^b)</td>
<td>42/89 (47)</td>
<td>15/89 (17)</td>
</tr>
<tr>
<td>New York</td>
<td>37/96 (39)</td>
<td>33/96 (34)</td>
</tr>
<tr>
<td>Tennessee</td>
<td>34/98 (35)</td>
<td>29/98 (2)</td>
</tr>
<tr>
<td>Ontario, Canada</td>
<td>54/112 (48)</td>
<td>8/112 (7)</td>
</tr>
<tr>
<td>Total</td>
<td>272/636 (43)</td>
<td>104/636 (16)</td>
</tr>
</tbody>
</table>

\(^a\) Samples were obtained from persons in the Houston metropolitan area as part of a pneumococcal carriage study.

\(^b\) Samples were obtained from noncase family members enrolled in a study examining the human genetics of susceptibility to tuberculosis, in Houston.

The presence of Sic antibodies in human nasal wash specimens.

The observations that Sic variants are selected on the mucosal surface and that Sic is important in persistence in the mouse upper respiratory tract indicate that selective pressure can be exerted at the mucosal surface. IgA is the most abundant immunoglobulin present on mucosal surfaces, and IgG also is present [20]. To test the hypothesis that Sic-specific immunoglobulin is present in host secretions, we examined nasal wash samples from 119 healthy young adult volunteers from the Houston metropolitan region who had provided the serum samples for Sic and M1 serum IgG measurements. Sic-specific and total IgA and IgG were measured by kinetic ELISA. In total, 113 nasal wash specimens contained detectable levels of IgA (>1.6 µg/mL), and 12 samples had Sic-specific IgA concentrations below detectable levels (<60 ng/mL), which were assigned a value of 30 ng/mL. The range of Sic-specific IgA concentrations was 82–803 ng/mL in the remaining 101 samples. Ninety-three samples had detectable levels of total IgG (>0.6 µg/mL). Of the 93 samples with detectable total IgG, 16 were assigned a value of 12.5 ng/mL because of below-detection levels (<25 ng/mL) of Sic-specific IgG. IgG levels were 28–75 ng/mL in the remaining 77 samples. These results demonstrate that humans commonly have mucosal antibodies that react with Sic. There was no significant difference in the levels of Sic-specific IgG present in nasal washes (expressed as a percentage of total IgG) between persons who possessed or lacked specific serum antibodies to Sic as assessed by the ELISA values of a 1:1000 dilution of serum (P > .05; figure 2). In contrast, the levels of Sic-specific IgA were significantly higher in subjects who had specific serum antibodies to Sic (P = .002; figure 2). Together, these results indicate that persons with no history of invasive

Persistence of Sic and M1 antibodies in serial serum samples.

The human serologic data indicated that antibodies directed against Sic were more prevalent than antibodies against the amino terminus of M1. One explanation is that anti-Sic antibodies persist longer in humans than anti-M1 antibodies. To test this hypothesis, we studied antibody levels to these 2 proteins in serial serum samples obtained from healthy adults. The interval between paired serum samples was 3–18 years (mean, 13 years). Sixteen of the 61 persons had anti-Sic antibody in their first serum sample, and there was no significant decrease in antibody levels over the time frame studied (figure 1). Consistent with the analysis of the 636 serum samples, far fewer persons had antibody directed against the amino terminus of M1 protein. Only 2 of the 61 subjects in the cohort had serum antibody. The interval between serum pairs for these 2 persons was 9 and 17 years. Their serum samples did not contain detectable anti-Sic antibodies. There was no significant decrease in anti-M1 antibody levels over the 9-year period, but there was a decline of >50% in antibody levels over the 17-year period. Thus, although the sample size is very small, these data are consistent with the hypothesis that the higher prevalence of Sic antibodies in human populations is the result of a preferential decline over time in the level of M1 serum antibodies relative to Sic antibodies.

Persistence of Sic antibodies in human nasal wash specimens.

The observations that Sic variants are selected on the mucosal surface and that Sic is important in persistence in the mouse upper respiratory tract indicate that selective pressure can be exerted at the mucosal surface. IgA is the most abundant immunoglobulin present on mucosal surfaces, and IgG also is present [20]. To test the hypothesis that Sic-specific immunoglobulin is present in host secretions, we examined nasal wash samples from 119 healthy young adult volunteers from the Houston metropolitan region who had provided the serum samples for Sic and M1 serum IgG measurements. Sic-specific and total IgA and IgG were measured by kinetic ELISA. In total, 113 nasal wash specimens contained detectable levels of IgA (>1.6 µg/mL), and 12 samples had Sic-specific IgA concentrations below detectable levels (<60 ng/mL), which were assigned a value of 30 ng/mL. The range of Sic-specific IgA concentrations was 82–803 ng/mL in the remaining 101 samples. Ninety-three samples had detectable levels of total IgG (>0.6 µg/mL). Of the 93 samples with detectable total IgG, 16 were assigned a value of 12.5 ng/mL because of below-detection levels (<25 ng/mL) of Sic-specific IgG. IgG levels were 28–75 ng/mL in the remaining 77 samples. These results demonstrate that humans commonly have mucosal antibodies that react with Sic. There was no significant difference in the levels of Sic-specific IgG present in nasal washes (expressed as a percentage of total IgG) between persons who possessed or lacked specific serum antibodies to Sic as assessed by the ELISA values of a 1:1000 dilution of serum (P > .05; figure 2). In contrast, the levels of Sic-specific IgA were significantly higher in subjects who had specific serum antibodies to Sic (P = .002; figure 2). Together, these results indicate that persons with no history of invasive
GAS disease can have both mucosal and systemic responses to Sic.

Degradation of Sic by the streptococcal cysteine protease. The observed variability in human antibody prevalence to Sic and M1 suggests there may be quantitative differences in expression or antigenicity of these 2 proteins in vivo. Production of Sic and M protein in vitro is controlled by Mga, a positive-acting gene regulator [21, 22]. However, nothing is known about regulation of sic and emm gene expression in vivo in humans. Modifications to proteins during infection could result in altered antigenicity. One mechanism by which Sic and M1 proteins could be modified in vivo is by proteolytic cleavage. In this regard, virtually all isolates of GAS express an extracellular cysteine protease known as pyrogenic exotoxin B or SpeB [23]. SpeB can cleave many secreted and cell-surface proteins of GAS in vitro, including streptolysin O, C5a peptidase, and M protein [24–26]. We found that purified SpeB also extensively degraded Sic in vitro (figure 3). Full-length (undegraded) Sic was detected only when a molar ratio of SpeB to Sic of 1:1000 was used (figure 3). These results demonstrate that Sic is very susceptible to degradation in vitro by small amounts of catalytically active SpeB cysteine protease.

Linear B cell epitope mapping of Sic. Sic protein variation could result in escape from immune surveillance, if the observed structural changes result in altered antigen-antibody recognition. If Sic variants are antibody escape mutants, then it is expected that host antibody would be commonly directed against the highly variable regions of Sic identified by the sic gene sequencing studies [8, 10]. Linear B cell epitope mapping was used to test this hypothesis. Overlapping synthetic peptides that span the entire mature Sic1.01 protein (281 aa) were used. The Sic1.01 variant was selected for analysis because it is the most frequently identified variant in M1 GAS isolates [8, 11].

We analyzed 29 serum samples randomly chosen from the 272 found to have specific antibody to Sic. Ten linear epitopes were recognized by ≧50% of the serum samples analyzed (figure 4). These linear epitopes are located in regions of Sic that commonly have insertions, deletions, or amino acid replacements (figure 5). Of note, only 8 of the 29 serum samples had reactivity against peptides corresponding to aa 199–208, a region of the Sic protein with a large number of amino acid replacements [8, 10]. To determine whether the relative lack of antibody reactivity to peptides corresponding to this region was an artifact of an inadvertent and unrecognized bias in the 29 serum samples chosen for analysis, the remaining 243 serum samples with anti-Sic antibodies were screened by ELISA against peptides 67–71 that spanned aa 193–219. Only 18 (7%) of the 243 serum samples had reactivity against these 5 peptides; the peak of reactivity for all 18 these was peptide 69 (SDWGQS-EDTPRPSE).

It is possible that the region represented by peptide 69 is part of a discontinuous epitope. If so, we anticipate that these 18 serum samples would react nonrandomly with another region of Sic that participates in formation of the discontinuous epitope. To test this possibility, the reactivity profiles of these serum samples...
Figure 4. Linear B cell epitopes identified in streptococcal inhibitor of complement (Sic) by human serum samples. Twenty-nine serum samples were screened by ELISA against a synthetic peptide library of 90 overlapping 15mers corresponding to the mature Sic1.01 variant. Frequency of reactivity was determined for each peptide by dividing the no. of serum samples positive for specific peptide by total samples. Nos. above vertical bars denote linear B cell epitopes identified frequently by serum samples. Schematic at top indicates position of repeat regions in Sic relative to peptide no. R1, R2, and R3, repeat regions; N, amino terminus; C, carboxy terminus. Sales were determined for the complete set of 90 Sic peptides. However, the profiles for the 90 peptides did not reveal non-random reactivity with other Sic peptides, a result suggesting that the amino acids in peptide 69 are not part of a discontinuous epitope (data not shown). Taken together, the data are consistent with the hypothesis that host antibody participates in selection of new Sic variants.

Phage display mapping of Sic epitopes. One concern with linear epitope mapping is that it does not accurately identify epitopes in the context of a folded protein with higher order structure. To address this concern, phage display was used to determine whether the regions of Sic identified by the overlapping peptide method would also be recognized by this method. The issue was first tested with a mouse MAb raised against Sic1.01 that has a minimal epitope of DWPEDDW. A total of 27 phage inserts were characterized, and the data showed that the common region identified in the inserts encoded a region with the amino acid sequence DDW (data not shown). Thus, these results validated the phage display strategy.

We next used 2 human serum samples to screen the Sic1.01 phage library. Thirty phages were selected by the human serum samples, and the sic inserts were sequenced. There was a strong correlation between the epitopes identified by the linear epitope mapping and phage display methods (figure 6). Together, the results indicate that the Sic epitopes identified by the linear epitope method accurately reflect the regions against which humans make antibodies to Sic.

Linear B cell epitope mapping of the amino terminus of M1.0. To date, 37 M1 variants have been identified that are characterized by single amino acid replacements and small deletions or insertions in the amino terminal variable region [4, 8, 11, 13]. Although these types of structural changes can alter antibody recognition of the M1 protein [13], linear B cell epitopes in this region have not been systematically characterized. To define the linear regions of M1 that are recognized by human serum antibodies, epitope mapping was conducted with 59 overlapping synthetic peptides that span the amino terminal variable region of the M1.0 protein. Nine serum samples selected from the 104 identified to contain specific antibody to M1 were tested by ELISA against the overlapping peptides. Analogous to the results for Sic, the linear M1 epitopes mapped to the highly variable region of the M1 amino terminus containing many amino acid replacements and insertions and deletions (figure 7).

Discussion

Molecular insights into the development and perpetuation of microbial epidemics of human disease are limited. The studies reported here were stimulated by the discovery that epidemics of serotype M1 GAS invasive infections are not mono- or pauciclonal, as long thought [8]. Rather, they are characterized by a remarkably diverse array of Sic variants that are rapidly selected on the mucosal surface of the mammalian host. The
Figure 5. Human antibodies to streptococcal inhibitor of complement (Sic) protein react with linear epitopes in Sic regions that are polymorphic in natural populations. Amino acid sequence of mature Sic1.01 protein is shown in segments defined by sequence features identified in [1]. Amino acid residues beneath the Sic1.01 sequence are amino acid replacements identified in a database of 296 sic alleles (GenBank accession nos. AF232306–AF232601). Variant amino acids recently identified in studies of Sic variation in Japan [12] also are included. Underlined residues correspond to regions in Sic characterized by many insertion and deletion events. Circled nos. indicate linear epitopes identified by human serum samples and correspond to nos. above vertical bars in figure 4. Red-colored residues delineate limits of each epitope.

overall goal of the present studies was to provide serologic data bearing on the host factors that may contribute to selection of new Sic variants.

Previous data [8] suggested a model in which Sic is rapidly diversified on host mucosal surfaces as a consequence of selection by acquired host immunity, perhaps antibody mediated. The identification of specific anti-Sic serum antibody in 43% of 636 persons clearly indicates that antibody against this GAS virulence factor is widespread in diverse human populations. Inasmuch as study subjects had no known history of invasive GAS infections, we conclude that Sic is commonly produced by the organism during mucosal interactions. The presence of Sic-specific IgA and IgG in most human nasal wash samples also is consistent with this idea. Moreover, these results place antibodies that recognize Sic at the mucosal site where selection of new variants occurs.

The linear B cell epitope mapping and phage display data are also consistent with and strengthen the hypothesis of antibody-mediated Sic diversification. Importantly, virtually all highly polymorphic regions of Sic were identified as epitopes commonly recognized by host antibodies. The relative lack of reactivity to Sic aa residues 193–219 was surprising, given the abundance of single amino acid replacements identified in this region. This suggests that the region is part of a discontinuous epitope or has functional importance unrelated to antibody interactions. However, epitope mapping of the entire Sic protein with the serum samples containing antibodies that react with the peptides comprising aa 193–219 did not reveal data consistent with the discontinuous epitope hypothesis. As a consequence, we speculate that the amino acid polymorphisms identified in this region arise by selection that does not directly involve host antibody. Taken together, our data are consistent with the idea that variation in Sic is shaped by a combination of immune evasion plus functional diversity.

Linear epitope mapping of the M1 protein amino terminus also found that antibodies are directed toward the region of this molecule characterized by amino acid polymorphisms. For reasons that are not understood, although there are many M1 variants, none increase in frequency in any of the geographic regions studied [8]. Given that most nucleotide substitutions in the emm1 gene result in amino acid replacements, natural selection also apparently is operating to shape variation in this molecule.

We previously hypothesized [8] that the selective pressure to
Figure 6. Comparison of streptococcal inhibitor of complement (Sic) epitopes identified in human serum samples by linear epitope mapping and phage display methods. Two serum samples (A and B) were screened by ELISA against a synthetic Sic1.01-specific peptide library to identify linear epitopes. These serum samples were also used to select Sic1.01-specific phage, as described in Materials and Methods. Results of phage display analysis are shown at the top of each panel. Nos. in parentheses represent no. of phage selected with specific insert. Nos. adjacent to each vertical line identify range of amino acid residues in mature Sic1.01 protein represented in phage insert. Vertical lines delineate limits of each phage insert with respect to linear epitope map of serum samples shown at bottom of each panel. OD$_{405}$, optical density$_{405}$.

Varying Sic exceeds the pressure to alter M1 protein. Several observations are consistent with this idea. First, a far larger percentage of subjects had serum antibody that reacted with Sic than the amino terminus of M1 protein. Second, anti-Sic antibody was also present in nasal wash specimens at high frequency. Third, we found that no significant decrease in anti-Sic serum antibody levels over about a decade, whereas in 1 of 2 persons, anti-M1 protein antibody levels decreased by 50%. Although the sample size was very small, we note that, in a study of the persistence of type-specific antibodies, Lancefield [27] was unable to detect M1-specific bactericidal antibodies in 2 persons 16 and 31 years after exposure to GAS. Similarly, Quinn et al. [28] observed that only 6 of 46 children who had GAS-positive throat cultures had type-specific bacteriostatic M
Figure 7. Human antibodies identify linear epitopes in variable regions of amino terminal region of protein M1 that arise in humans. Top. Sequence of amino terminal hypervariable region of M1.0 protein (GenBank accession no. X07933). Asparagine at position 10 represents the beginning of the mature protein. Each amino acid residue corresponds to the 7th residue of the M1-specific peptide aligned beneath it in the graph below. Final 11 peptides overlap by 12 amino acids. Amino acid residues beneath M1.0 sequence are amino acid replacements identified in database of 37 emm1 alleles (authors’ unpublished data and [4, 11]). Bottom. Graph of frequency of reactivity of 9 human serum samples for each M1.0-specific overlapping peptide, as determined by dividing the no. of serum samples positive for a specific peptide by the total number of samples.

protein antibodies after 1 year. In addition, Flores et al. [29] studied 68 children with upper respiratory tract and skin infections and reported M-protein serotype-specific differences in the development of functional (bactericidal) type-specific antibodies, suggesting that distinct M types vary in their ability to elicit an immune response, protective or otherwise. Hence, the difference in frequency of occurrence of antibody to the amino terminus of M1, compared to Sic, in human serum may reflect qualitative or quantitative differences in immune system interaction with these 2 proteins.

It may also be that Sic and M1 proteins differ in level of in vivo production. Akesson et al. [1] reported that Sic was present in substantial amounts in the culture supernatants of an M1 strain grown in vivo. However, there has not yet been a direct comparison of the level of production of Sic and M1 protein among many serotype M1 strains. A related possibility is that humans are exposed to antigens that elicit antibodies that cross-react with Sic more frequently than they are exposed to antigens that elicit M1 cross-reacting antibodies. However, Sic has no substantial homology with other proteins in available public databases. We also note that the sic gene is present largely in serotype M1 organisms and apparently is not widely distributed among GAS strains [1].

Considerable effort has been expended in recent years to identify host immunologic factors that contribute to susceptibility to invasive infections caused by serotype M1 GAS strains [30–35]. Investigators have reported [30, 33] that healthy persons or patients with uncomplicated infections have higher serum antibody levels against M1 antigen than do patients with bacteremia or fatal infections. These observations, together with the generally accepted notion that M-type–specific opsonic antibodies are protective [27], have led to the concept that low or lack of antibody against M1 protein is a risk factor for development of invasive disease caused by strains expressing this antigen. However, relatively little information is available about the prevalence of anti–M1 antigen antibody in healthy persons living in diverse localities. We found a relatively low prevalence of antibody against M1 antigen in humans in all 5 diverse localities studied. Notwithstanding the potential effect of an amnestic response and data indicating that antibodies against non–M protein factors influence host-pathogen interactions [36–41], our data are consistent with the hypothesis that a rel-
atively large numbers of humans are at risk of developing M1 infections, including severe invasive episodes. Hence, continued efforts directed toward developing a human GAS vaccine are warranted.

In summary, anti-Sic antibody is widespread in human populations, both in serum and nasal wash specimens. Human anti-Sic antibody is directed against virtually all regions of Sic that are highly polymorphic in natural populations. Our results support the concept that selection of Sic variants occurs on mucosal surfaces by a mechanism that involves acquired host antibody. Although many pathogen and host factors participate in epidemics caused by GAS, on the basis of data presented in this report and in other recent publications [4, 8, 10, 14, 42], insight into the molecular adaptations that specifically assist M1 strains to survive host defenses, become abundant, and cause widespread human disease are now beginning to emerge.

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References