Variable Susceptibility to Opsonophagocytosis of Group A Streptococcus M-1 Strains by Human Immune Sera

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Immunity to group A streptococci (GAS) is thought to be related to the acquisition of type-specific antibody directed against the M protein. However, recent work suggests that immunity may only be strain and not M-type specific. Therefore, susceptibility of 70 different GAS M-1 strains to opsonization and killing by convalescent sera was compared by using a highly sensitive chemiluminescence assay and by standard bactericidal assay. Sequencing of the emm1 gene in 10 strains with variable susceptibility to opsonization revealed 100% homology in 9 strains. Several substitutions in the N-terminal and 2 in the A repeat regions of strain CS-190 were associated with profound resistance to opsonization. Thus amino acid substitutions within different regions of the M-1 protein molecule may adversely affect opsonization by immune sera. In addition, non-M protein factors from identical M types influence susceptibility to phagocytosis. These findings may in part explain the persistently high prevalence of M-1 strains worldwide over the last 15 years.

Group A streptococci (GAS) continue to cause mild-to-severe human infections worldwide. The organism is responsible for many common suppurative infections such as pharyngitis, tonsillitis, scarlet fever, pneumonia, impetigo, cellulitis, erysipelas, and lymphangitis, as well as nonsuppurative sequelae such as rheumatic fever and poststreptococcal glomerulonephritis. In the United States, the incidence of rheumatic fever has declined over the last 40 years [1]; however, recent outbreaks of rheumatic fever and the emergence of invasive GAS infections [2] and streptococcal toxic shock syndrome [3, 4] have refocused attention on the need for effective control and treatment measures.

Although Streptococcus pyogenes possesses several virulence factors, such as streptolysin-O, hyaluronidase, streptokinase, C5a peptidase, and pyrogenic exotoxins, it is the M protein, a surface component, that renders the organism resistant to phagocytosis [5]. The M protein’s α-helical–coiled coil secondary structure is rare in bacteria, but it shares physicochemical properties and sequence homology with some mammalian fibrillar proteins such as myosin heavy chain and α-tropomyosin [5]. The homology with mammalian proteins provides the basis for cross-reactive antibodies against sarcolemmal membrane proteins of human myocardium [6].

After a GAS infection, type-specific opsonic antibodies appear in 4 weeks and persist for 4–32 years [7, 8]. These antibodies are expected to protect against future infections by homologous serotypes [9]. In experimental models using rabbits, the development of type-specific antibodies after immunization with crude or purified M protein extracts is detectable by 14 days. Passive transfer of serum from the immunized rabbits protects mice against >1000 lethal doses of the homologous strain but does not protect against heterologous GAS strains [10]. Thus, protection against infection has been correlated with the presence of type-specific opsonic antibodies against M protein [5].

DNA sequencing of numerous emm genes has shown that the 3' end encoding the carboxy terminus is highly conserved among different M proteins. Conversely, the amino-terminal segments are highly variable. This variability in the N-terminus forms the basis for serologic typing of GAS according to M type [11]. The N-terminal or A-repeat regions of the M protein are the sites for attachment of type-specific opsonic antibody [5, 12], and some studies have demonstrated that 1 epitope
resides within the first 10 amino acids (aa) of the N-terminus [13].

Phagocytosis, a primary host defense mechanism against invading bacteria, is performed largely by professional phagocytes, such as polymorphonuclear leukocytes, monocytes, and macrophages. Opsonins, such as immunoglobulin and complement, facilitate phagocytosis and killing of microorganisms [14]. Established assays of opsonophagocytosis of GAS have used quantification of bacterial killing, such as the indirect bactericidal test (IBT) [15] or direct counting of GAS within phagocytes [16]. In general, phagocytosis can also be quantified indirectly by measurement of the metabolic and chemical products associated with the engulfment and killing of the opsonized particles by leukocytes [17–19]. The photon emission by these metabolic and chemical products can be amplified by three orders of magnitude by luminol, which allows quantification of oxidative activity (chemiluminescence [CL]) using relatively few phagocytes [18].

The objectives of this study were to compare the susceptibility to opsonization of 70 different M-1 strains against convalescent sera, using a rapid, sensitive, and reproducible whole blood CL assay [20], and to determine whether differences in opsonization are related to differences in the \textit{emm} gene.

**Materials and Methods**

**Strains and cultures.** For this study we used 70 serotype M-1 GAS strains from the collection of one of the authors (D.L.S.). Most of the strains were associated with invasive cases of GAS infection and were isolated from a sterile body site (\(n = 59\)), Germany (\(n = 6\)), Japan (\(n = 2\)), The Netherlands (\(n = 2\)), and Switzerland (\(n = 1\)) from 1988 to 1997. An M-1 strain (CS-190), which possesses nucleotide substitutions in the N-terminal nonrepeat region and altered type-specific antibody recognition [12] (provided by P. Cleary, Department of Microbiology, University of Minnesota), was also included. M-3 and M-11 GAS strains were used as negative controls. All strains were typed by 2 of us (E.L.K. and D.R.J.) at the World Health Organization (WHO) Streptococcal Reference Laboratory, University of Minnesota.

One colony of each strain was grown in 9 mL of Todd Hewitt broth plus 1% neopeptone (THBN; Difco, Detroit) overnight (18 h) at 37°C in 5% CO₂. The overnight broth culture was used to inoculate 90 mL of previously warmed THBN and was incubated for 4 h at 37°C in 5% CO₂ with shaking (100 rpm) to obtain a logarithmic phase growth. After the cultures were centrifuged at 1700 \(\times\) g for 15 min and washed twice in 10 mL of ice-cold Dulbecco’s PBS (Sigma, St. Louis), the bacterial pellet was resuspended in PBS. Each bacterial suspension was placed in an open 100-mm sterile polystyrene petri dish and was killed either by exposure to 254 nm of UV light for 1–4 h or by heating at 56°C for 1 h. All CL assays were done using bacterial suspensions of each strain at a standardized concentration corresponding to 0.5 \(A_{540nm}\).

**Phagocytes.** For CL experiments, whole blood from a single normal donor (A.V.) who lacked opsonic antibody against M-1 and M-3 GAS strains, as demonstrated by the blood rotation test (growth index \(\geq 32\)) [21], was anticoagulated by using standard EDTA-treated vacuum tubes (Becton Dickinson, Rutherford, NJ). The blood sample was placed on a blood rotator at room temperature (23°C–24°C) and was used within 5–30 min. To compare results of assays done on different days, a white blood cell count and differential were done on blood from the same normal donor, and results were expressed as counts per minute (cpm/phagocyte).

**Serum samples.** A normal serum sample was obtained from a donor who lacked opsonic antibodies against M-1 and M-3 GAS strains in the indirect bactericidal test [20]. The bactericidal activity of “normal” serum (from A.V.) was similar to the activity of a normal rabbit serum used as the negative control for M-1 and M-3 GAS strains at the WHO Streptococcal Reference Center in Minnesota [15]. A convalescent serum sample from a donor (D.R.J.) with a history of streptococcal pharyngitis due to an M-1 strain was used as a positive serum. This subject had not received antibiotics or drug treatment in the previous 2 weeks. The bactericidal activity of this serum against an M-1 strain was similar to the bactericidal activity of rabbit anti-M-1 antiserum used as positive control (WHO standard anti-M-1 serum) in the IBT [21]. Serum samples were maintained in cryogenic tubes at \(-70°C\) until used.

**CL opsonophagocytosis assay.** Opsonization was done as previously described [20]. In brief, a mix of killed bacteria and diluted convalescent or normal serum was incubated at 37°C for 30 min. After wash, the samples were spun and the supernatant discarded [20]. Bacteria were resuspended in PBS, and a prewarmed balanced salt solution containing luminol, Ca²⁺, Mg²⁺, and glucose [22] was added to each tube. The samples, in duplicate, were placed in the luminometer at 37°C, and 100 \(\mu\)L of diluted blood from a normal donor was automatically injected into each tube.

The CL measurements were made every 0.5 min during a 20-min incubation. Results were expressed as cpm per phagocyte as an accumulated or integral value [20]. A photon standard was used to standardize assays between runs. Additional controls were as follows: (1) M-1, M-3, and M-11 bacterial suspensions incubated with PBS; (2) M-1, M-3, and M-11 strains incubated with normal serum; (3) M-3 and M-11 GAS strains incubated with a heterologous convalescent anti-M-1 serum; and (4) 50 \(\mu\)L of complement-opsonized zymosan [22].

Testing the effect of complement in the opsonization of GAS by the CL assay: To test the possible effect of complement in the opsonization of GAS, a heat-killed M-1 strain was incubated with either PBS, 20 \(\mu\)L (3 U CH₅₀) of guinea pig complement (Sigma), anti-M-1 convalescent sera, heat-inactivated anti-M-1 convalescent sera, or heat-inactivated anti-M-1 sera plus guinea pig complement. After 30 min, the organisms were washed and used immediately as described above.

**IBT.** The IBT was done using heparinized whole blood from a normal donor by the method of Lancefield [15] as modified by Johnson et al. [21] to include a preopsonization step. As in other laboratories [21], we calculated the bactericidal index (BI) and the corresponding logarithm base 2 of the BI (BLog₂) to facilitate comparisons. A BI \(\geq 32\) or the corresponding BLog₂ \(\geq 5\) was considered significant bacterial killing. The resulting BI values were also interpreted by the scale described by Stollerman et al. [23]. Standardized normal and anti-M-1 rabbit sera used as controls were donated by D.R.J. and E.L.K.
Figure 1. Chemiluminescence (CL) assay. Heat-killed M type 1 group A streptococci (GAS) were incubated 30 min at 37°C with PBS, convalescent anti-M-1 (homologous) serum, or convalescent anti-M-1 serum that had been adsorbed against M-1 strain (adsorbed). GAS were washed once and resuspended in luminol-containing salt solution. Diluted whole blood from donor lacking antibody to M-1 GAS (100 μL) was added, and CL responses were measured for 20 min. Results are mean cpm/phagocyte of duplicate samples ± SD. To verify specificity of assay, M type 11 strain incubated with convalescent anti-M-1 serum (heterologous), was included. Complement-opsonized zymosan (C-op-zym) was added as positive control.

Figure 2. Effects of complement on group A streptococcus (GAS) opsonization. M-1 GAS strain (96/004) was incubated for 30 min at 37°C with PBS, guinea pig complement (3 U CH50), anti-M-1 convalescent serum, heat-inactivated convalescent anti-M-1 serum, or heat-inactivated anti-M-1 serum plus complement. Organisms were washed once and resuspended in luminol, and diluted whole blood from donor lacking antibody to M-1 GAS was added. Chemiluminescence responses of duplicate samples, measured for 20 min, are expressed as mean cpm/phagocyte ± SD.

Sequencing of the emm gene. The emm gene of M-1 GAS strains was sequenced at the University of Oklahoma Advanced Center for Genome Technology. In brief, chromosomal DNA was isolated from overnight cultures of GAS grown in 10 mL of THY broth by a modification of the method of Pitcher et al. [24]. Bacteriophage C1 lysin (10 U) was used to supplement the lysozyme specified in the original protocol to greatly enhance degradation of the cell walls. The variable portion of emm was amplified by primer pairs described elsewhere (primer 1, 5'-TATT[GC]GCTTAGAAAATTA; primer 2, 5'-GCAAGTTCTTCAGCTGT) [25]. By using the optimized conditions of Beall et al. [26], polymerase chain reaction products were cloned into the TA cloning vector pGEM-T Easy (Promega, Madison, WI) according to the manufacturer’s recommendations. Subsequently, the inserts were sequenced after purification of the plasmids by commercial kit (Qiagen, Valencia, CA). Sequencing was done by automated DNA sequencer (model 391; Perkin-Elmer, Foster City, CA). Multiple DNA sequence alignment was done using the program CLUSTAL-X [27] on a Macintosh Power PC (model 7600).

Results

Type specificity of the CL assay. A representative CL assay, depicted in figure 1, demonstrates type specificity in 3 ways: a high phagocytic response after incubation of an anti-M-1 convalescent serum with a homologous M-1 strain, a dramatic reduction in the phagocytic response after adsorbing this serum against a homologous M-1 strain, and low phagocytic activity after incubation of the same convalescent serum with a heterologous M-3 GAS strain. The nonopsonized M-1 strain (incubated with PBS) showed a baseline phagocytic response. To verify the responsiveness of phagocytes in diluted whole blood, a control using complement-opsonized zymosan [22] was also included.

Effect of complement in CL assay opsonization of GAS. The CL responses of the phagocytes to heat-killed M-1 GAS incubated with PBS (mean, 162 cpm/phagocyte) or complement (mean, 98 cpm/phagocyte) were not significantly different ($P = .08$; figure 2). In contrast, the CL response of the phagocytic cells after challenge with M-1 GAS incubated with a convalescent anti-M-1 serum was significantly higher (mean, 863; $P = .003$). No significant decrease in CL occurred after heat inactivation of the convalescent sera ($P = .17$). Similarly, addition of guinea pig complement to heat-inactivated convalescent anti-M-1 serum did not increase CL responses compared with heat-inactivated serum alone (figure 2). In these experiments, the CL assay showed no significant role for complement in the opsonization of M-1 GAS.

CL assays. Incubation of each of the 70 M-1 strains with a single convalescent serum (D.R.) generated a heterogeneous phagocytic CL response in a range of 47–477 cpm/phagocyte (mean, 310; SD, 75). The CL responses to these strains were distributed as follows: 51 strains (73%) were within ±1 SD of the mean; 16 (23%) were between SDs 1 and 2 on both sides.
of the mean; and 2 (3%) were between SDs 2 and 3 on both sides of the mean. The phagocytic responses showed a normal distribution (Kolmogorov-Smirnov = 0.067; P > .200). Heterologous M-3 and M-11 control strains were placed after the third SD below the mean (figure 3).

Experiments were repeated using 2 additional convalescent anti–M-1 sera (A.B., K.F.). These sera were tested against representative M-1 strains that showed variable susceptibility to opsonization with the first convalescent (D.R.) anti–M-1 serum (figure 3). We used a well-opsonized strain (96/004) and a poorly opsonized strain (CS-190) in the CL assay (table 1). Significantly higher opsonization of the M-1 strain 96/004 than of the M-1 CS-190 strain (P < .05) was again achieved by both additional convalescent anti–M-1 sera (table 1).

**IBT.** A subset of 10 strains from the 70 M-1 strains tested above was selected to represent a spectrum of opsonophagocytic susceptibility (figure 3). For each strain, a growth index >32 was required to demonstrate that the strain produced sufficient quantities of M protein to resist phagocytosis in normal serum (data not shown). The bactericidal activity, as measured by the IBT of a single anti–M-1 convalescent serum against each of the 10 homologous M-1 strains, was heterogeneous, with a BLog$_2$ of 1.5–8.9. Similarly, this same convalescent

### Table 1. Variability in opsonization of M-1 group A streptococci (GAS) by homologous convalescent sera is not donor-specific.

<table>
<thead>
<tr>
<th>Source of anti-M-1 sera</th>
<th>Opsonophagocytosis (mean cpm/phagocyte) of Streptococcus pyogenes strains</th>
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<tbody>
<tr>
<td></td>
<td>M-1 96/004</td>
<td>M-1 CS-190</td>
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<tr>
<td>Donor 1</td>
<td>433</td>
<td>119</td>
</tr>
<tr>
<td>Donor 2</td>
<td>328</td>
<td>112</td>
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<tr>
<td>Donor 3</td>
<td>426</td>
<td>247</td>
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NOTE. Convalescent serum samples were from 3 volunteers who had prior M-1 GAS infections. M-1 strains (96/004 and CS-190) with widely divergent sensitivities to opsonization by convalescence anti-M-1 serum and GAS M-3 strain (88/003) were incubated with each convalescent serum sample for 30 min at 37°C (see Materials and Methods).

*Chemiluminescence assay responses between 2 M-1 strains in each serum; P < .05 considered significant by Student’s t test.

Figure 3. Chemiluminescence (CL) responses (●) to 70 M-1 group A streptococcus (GAS) strains incubated with 1 convalescent anti-M-1 serum, washed once, and resuspended in luminol-containing salt solution. 100 µL of diluted whole blood from donor lacking antibody to M-1 GAS was added, and CL responses were measured for 20 min. Negative controls, heterologous strains M-3 and M-11 (▲), appear on left of curve >3 SD below mean. Kolmogorov-Smirnov test showed normal distribution of results. 10 strains from this distribution (□) were selected at random for further analysis and for sequencing of *emm* gene.
Figure 4. Schematic representation of mature M-1 protein from *Streptococcus pyogenes*. M protein is helical coiled coil consisting of N-terminal hypervariable region; A repeat, C repeat, and D regions; and anchor domain. Opsonic antibodies bind to hypervariable and/or A-repeat regions.

**DNA sequencing.** In order to learn the reason for heterogeneity in opsonophagocytosis demonstrated in both CL and bactericidal assays, the 10 M-1 strains were subjected to *emm* gene sequencing. Analysis of the predicted aa sequence of the mature M protein demonstrated a common pattern composed of the N-terminal hypervariable, A repeat, B repeat, C repeat, and anchor regions (figure 4). Six strains demonstrated 100% homology at the deduced aa sequence level from the N-terminus through the three A-repeat regions (figure 5). One strain (95/007) possessed a single aa substitution localized at the end of the A1 repeat region (figure 5). Of note, this strain was well opsonized in both the bactericidal (BILog2 = 6.9) and the CL assays (figure 3; >1 SD above the mean).

Finally, strain CS-190 had 2 aa substitutions localized in the A3 repeat region and several others in the N-terminus (figure 5). This strain had the lowest susceptibility to opsonization in both bactericidal (BILog2 = 1.5) and CL assays (figure 3), with values near those of the M-3 and M-11 heterologous strains used as negative controls.

**Discussion**

During the past 7 decades, different approaches have been used to demonstrate the presence of type-specific opsonic antibodies against GAS. The bactericidal test was developed by Todd [28] and Lancefield [15] to demonstrate the presence and specificity of M antigens in GAS, as well as the presence of type-specific anti-M antibodies in convalescent sera. Although the bactericidal test remains the reference standard, it is cumbersome and time-consuming, taking up to 3 days to complete and requiring large amounts of undiluted serum (120 µL/tube). Also, the number of samples per run is limited, making this assay impractical for large-scale epidemiologic studies.

In this study, we used a highly sensitive, rapid, and simple whole blood CL assay, which was highly correlated with the IBT [20], to measure the dynamics of opsonization of M-1 strains by convalescent anti-M-1 sera. With this CL assay, we obtained a heterogeneous phagocytic response. To illustrate, the majority of strains caused a mean phagocytic response of 310 cpm/phagocyte. In contrast, 7 strains were poorly opsonized, with results >1 SD below the mean, and 1 (CS-190) was opsonized no better than heterologous strains belonging to M-3 and M-11 serotypes.

Traditionally, it has been assumed that after a GAS infection, the host will develop protective antibodies against all strains
Figure 5. Comparison of deduced amino acid (aa) sequence of several M-1 group A streptococcus (GAS) strains. Regions corresponding to N-terminal hypervariable region and A-repeat regions were selected for amino acid (aa) comparison. aa substitutions are shown in bold and underlined.

of the homologous serotype [9]. However, successful development of protective type-specific and other nonprotective antibodies by the host is not always observed after a GAS infection. Some studies found that antimicrobial treatment of GAS infections may interfere with or prevent the development of type-specific and anti-streptolysin O antibodies [7, 8].

In our study, despite the successful development of type-specific opsonic anti-M-1 antibodies from donors with histories
of M-1 GAS infections, these antibodies opsonized and facilitated the killing of different M-1 strains of GAS with highly divergent efficiency: The level of anti–M-1 antibody in a single serum sample was rated from low, “not significant” to “strongly positive” [23]. This heterogeneous activity was also not donor specific. The heterogeneous opsonic activity against GAS M-1 strains observed in the present work, as well as the findings of de Malmanche and Martin [29], who also found that typespecific opsonic antibodies opsonize some but not all M-1 strains, suggests that protection may be only strain and not type specific.

To investigate the molecular mechanism responsible for the heterogeneous CL response, we compared the deduced aa sequence of the N-terminal and the A-repeated regions of M proteins from GAS strains that elicited high, intermediate, or low phagocytic or bactericidal activity in the CL and bactericidal assays, respectively. Strain CS-190 had 2 aa substitutions in the A repeat and several in the N-terminal and was associated with the lowest CL response, supporting the notion that minor changes in the nucleotide sequence of the N-terminal [12] and A-repeated regions [30] alter recognition by type-specific opsonic antibodies. However, we also found that the wide spectrum in phagocytic and bactericidal activity of convalescent sera, and small-scale insertion produce size and antigenic variation in group A streptococcal M1 protein. Mol Microbiol 1993; 8:981–91. 13. Robinson JH, Kehoe MA. Group A streptococcal M proteins: virulence factors and protective antigens. Immunol Today 1992; 13:362–7. 14. Kalmar JR. Measurement of opsonic phagocytosis by human polymorphonuclear neutrophils. In: Clark VL, Bavoil PM, eds. Methods in enzymology. San Diego: Academic Press, 1994:108–19. 15. Lancefield RC. Differentiation of group A streptococci with a common R antigen into three serological types with a special reference to the bac-


