Determination of Group A Streptococcal Anti–M Type–Specific Antibody in Sera of Rheumatic Fever Patients after 45 Years

James F. Bencivenga, Dwight R. Johnson, and Edward L. Kaplan
Department of Pediatrics, University of Minnesota Medical School, Minneapolis

Group A streptococcal M type–specific protective antibodies—especially their persistence in humans—are incompletely understood. Such information is essential for understanding the epidemiology and pathogenesis of these infections and their sequelae and is equally crucial for producing a group A streptococcal vaccine. We studied 2 adults for type-specific antibody 45 years after they experienced documented rheumatic fever.

Studies by Wannamaker and colleagues [1, 2] in the early 1950s demonstrated the importance of M type–specific antibody in human resistance to infection with group A streptococci (GAS). On the basis of these and a few subsequent reports, efforts to develop GAS vaccines have largely been based on type-specific protective opsonic antibodies [3]. In 1959, Lancefield [4] described the persistence of type-specific opsonic antibodies in human sera for as long as 32 years. That report is a major basis for the belief that M protein–specific opsonic antibodies persist for extended periods of time, possibly even conferring lifelong type-specific immunity. To our knowledge, no additional information about the duration of type-specific immunity after natural human infection has since been published. Having identified two 56-year-old adults who had rheumatic fever at age 11 during a documented 1961 outbreak in Dickenson, North Dakota [5], we were able to examine their sera to investigate the persistence of M protein–specific opsonic antibody. The findings of the present study add to the sparse information about the duration of M type–specific immunity after natural infection. The conclusions have potentially significant implications for GAS vaccine strategies based on the hypervariable opsonic region of the M protein molecule [3].

Methods. Sera were obtained from two 56-year-old adults (D-1 and D-2), both of whom experienced documented rheumatic fever and were included in the published report of the 1961 outbreak (M.L. and R.F.) [5]. Serum specimens from subjects D-1 and D-2 were obtained after receipt of institutional review board approval and written informed consent.

The opsonic activity of these sera was determined using the bactericidal test, an in vitro test that determines type-specific antibody-dependent phagocytic killing of GAS actively growing in human blood. Both the classical Lancefield bactericidal method [6] and a published modification of that technique incorporating a preopsonization step [7–9] were used.

Briefly, the preopsonization method used stationary-phase GAS that were preincubated with the serum to be tested. After this preopsonization step, an aliquot of the GAS-serum mixture was added to freshly obtained and heparinized human whole blood. This mixture was then incubated, with constant mixing by end-over-end rotation for 3 h, as originally described by Lancefield [6]. To ascertain killing or inhibition of GAS growth during the test, viable colony counts on blood agar plates were performed for all GAS-serum combinations before starting and after completing the 3-h test.

Control sera used in all bactericidal tests included hyperimmune anti–M-5 and anti–M-24 rabbit sera, normal rabbit serum, and normal human sera.

Growth of GAS during the 3-h test period was calculated by dividing the posttest viable colony count by the corresponding pretest colony count, as described elsewhere [9]. The resulting growth index (GI) was then used to calculate the percent inhibition for each tested serum, as follows: \( \text{GI}^{\text{negative control}} - \text{GI}^{\text{serum}} \div \text{GI}^{\text{negative control}} \). As suggested in the report by Hu et al [3], >50% inhibition was considered significant. All experiments were repeated, and results were comparable.

All GAS strains and control sera came from our laboratory’s extensive collection. The M-5 strain (UMN 72501), isolated during the Dickenson rheumatic fever outbreak in 1961, was received from Zimmerman (one of the report’s authors) in 1972. The reference M-5 strain (UMN 71840) was obtained in 1971 from Maxted of the Public Health Service Laboratory in London. The reference M-24 strain (UMN 71694), used as the heterologous M type control strain, was obtained from Lancefield in 1971.

Results. GAS strains were originally characterized by clas-
sical M serotyping and were recently confirmed by emm gene sequence typing. The M-5 reference strain was identified as emm subtype 5.14. The Dickinson M-5 outbreak-associated strain proved to be a previously unrecognized emm sequence (subsequently designated emm 5.68) [10]. The 5.68 sequence differs from emm 5.14 by a single-base substitution that results in an amino acid change in the hypervariable region of the M protein molecule [10].

To detect possible non–type-specific opsonic activity in test sera, the M-24 GAS strain was included as a control in all tests. M-24 has long been uncommon in the United States, thus significantly reducing the probability of prior exposure for study subjects and controls. There was no inhibition of M-24 growth (ie, no bactericidal antibody activity) by either of the 2 subject’s serum or by the anti–M-5 rabbit serum (Table 1), whereas anti–M-24 rabbit serum resulted in complete killing of the homologous strain. Thus, no nonspecific bactericidal activity was detected.

The reference M-5 GAS was included in all bactericidal tests to detect M-5–specific bactericidal antibody. Serum from subject D-1 inhibited growth of the reference M-5 GAS strain by 66%, compared with control human serum or rabbit sera. In contrast, serum from subject D-2 showed no inhibition (killing) of the growth of the reference M-5 strain (Table 1).

Serum from subject D-1 (but not D-2) inhibited growth of the rheumatic fever outbreak–associated M-5 strain by 77%. This bactericidal activity against the rheumatic fever M-5 strain (emm 5.68) did not significantly differ from that observed with the genetically distinct reference M-5 strain (emm 5.14), suggesting that the structural differences in the M protein between these 2 strains did not influence susceptibility to serum opsonic antibodies (Table 1).

**Discussion.** Studies in the early 1950s initially demonstrated the important role played by M type–specific antibodies in resistance to infection caused by the homologous M serotype GAS in humans [1, 2]. However, the duration of persistence of these “protective” antibodies after natural infection in humans has not been comprehensively studied. The opportunity to evaluate the persistence of specific, opsonic, protective antibodies in 2 human sera collected 45 years after bona fide GAS infection resulting in rheumatic fever is unique, as far as we can determine. In fact, we know of no other attempts to confirm the 1959 report by Lancefield in which she demonstrated persistence in human sera 32 years after natural pharyngeal GAS infection.

We chose to use what we have reported to be a more sensitive preopsonization bactericidal method [7] after the classical Lancefield technique of indirect bactericidal testing yielded no significant inhibition of GAS growth by either subject’s serum. We were then able to confirm the presence of serum bactericidal antibody to the 1961 epidemic M-5 strain almost half a century after a natural pharyngeal GAS infection. The necessity of using the more sensitive assay suggests that perhaps only modest antibody levels were present. Given that protective levels of circulating opsonizing bactericidal antibody in humans have not been precisely defined, one can only speculate about their in vivo protective significance in humans.

We also confirmed the M serotype specificity of the circulating opsonic antibodies (subject D-1). Growth of both the M-5 rheumatic fever–associated strain and the M-5 reference strain were significantly inhibited by D-1 serum, whereas this serum resulted in no detectable inhibition of the growth of the reference M-24 strain. These findings indicate that nonspecific opsonizing antibodies (or other substances) were not likely responsible for the observed bactericidal activity against the M-5 strains.

Why only 1 of the 2 subjects who had confirmed rheumatic fever was found to have persistent opsonic activity against the specific outbreak-associated M-5 strain remains unanswered. However, our observed difference is consistent with our additional analysis of Lancefield’s 1959 report [4], which revealed no convincing correlation between “lower levels” of bactericidal activity and the time interval after natural pharyngeal infection.

The observed disparity might be related to the differences inherent in an individual’s immune-response capability. Ad-

**Table 1. Growth Index (GI), Bactericidal Index (BI), and Percent Inhibition of the Test and Control Sera after Incubation with Different Strains of Group A Streptococci (GAS)**

<table>
<thead>
<tr>
<th>Serum</th>
<th>M/emm type 24</th>
<th>Reference M-5 (emm 5.14)</th>
<th>Outbreak M-5 (emm 5.68)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GI</td>
<td>BI</td>
<td>Inhibition, %</td>
</tr>
<tr>
<td>D-1</td>
<td>50.4</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>D-2</td>
<td>54.0</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Positive control</td>
<td>0</td>
<td>...</td>
<td>100</td>
</tr>
<tr>
<td>Negative control</td>
<td>51.7</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

**NOTE.** The GI was calculated by dividing the posttest viable colony count by the corresponding pretest colony count [9]. The BI was calculated by dividing the growth index of the GAS strain in antibody-negative control sera by the growth index of that strain in the test serum [9]. Percent inhibition was calculated as follows: \(\text{Percent inhibition} = \left(\frac{\text{GI}_{\text{control}} - \text{GI}_{\text{test}}}{\text{GI}_{\text{control}}}\right) \times 100\). The >50% inhibition of the 2 M-5 streptococci with the D-1 serum suggests circulating opsonic antibodies.
ditionally, one cannot eliminate with certainty the possibility that the subject with “more” antibody (D-1) experienced additional exposure to this or another M-5 streptococcal strain during the intervening 45 years, thus “boosting” antibody levels.

Trials evaluating the efficacy of a multivalent GAS vaccine have been conducted; the preliminary outcomes have been encouraging [11]. One long-held concern regarding M protein–based vaccines has been whether adequate protection would be provided against variant strains within a given M serotype of GAS that show small changes within the M protein. Our data suggest that, although serum from subject D-1 did show slightly greater growth inhibition of the \textit{emm} 5.68 outbreak strain compared with the \textit{emm} 5.14 reference strain (77\% vs 66\%), we believe the difference was likely within the error limits of the technique. Additionally, hyperimmune rabbit serum against the \textit{emm} 5.14 strain effectively killed both M-5 strains. Considered together, these findings suggest that an individual with natural or vaccine-induced immunity to either 5.14 (currently thought to be the most common \textit{emm} 5 subtype in the US) or to 5.68 might possess cross-protection against the other subtype. This suggestion is in agreement with a recent report by Dale et al [12].

The duration of vaccine-induced protection relative to the duration of protection after natural infection is another issue that remains to be clarified. Our observations confirm and extend the early findings of Lancefield [4]. If circulating opsonizing antibody is a reliable predictor of protection, then protection may potentially last for decades after natural infection. Vaccine protection may be as long-lasting, but until more information is available from prospective human trials the duration of either natural or vaccine-induced protection against GAS infection requires further study.

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References