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ORIGINAL ARTICLE

Prospective Longitudinal Analysis of Immune Responses in Pediatric Subjects After Pharyngeal Acquisition of Group A Streptococci

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Background. Despite the significant burden of disease associated with infection by group A streptococcus (GAS), little is known about the human immune response to GAS antigens after natural infection.

Methods. We evaluated 195 serum samples obtained prospectively over a consecutive 24-month period from 41 pediatric subjects who experienced a new pharyngeal GAS acquisition. An enzyme-linked immunoassay was used to determine the kinetics and antigen specificity of antibodies against 13 shared GAS antigens and 18 type-specific M peptides. The majority of the antigens tested are currently being considered as vaccine candidates.

Results. Twelve M types of GAS were recovered from 41 subjects who experienced 51 new GAS acquisitions that elicited antibody responses against at least 1 of the 31 antigens tested (immunologically significant new GAS acquisitions). The immune responses to the 13 shared antigens were highly variable. Increases in antibody levels were detected against a mean of 3.5 shared antigens (range, 1–8). Antibody responses to the homologous M peptide were observed in 32 (63%) of the 51 episodes. Seven subjects acquired more than 1 M type of GAS. There were no new immunologically significant acquisitions of an M type against which the subject had preexisting antibodies to the homologous M peptide. Of the subjects with new GAS acquisition, 65% were asymptomatic, yet immune responses were detected against 1 or more GAS antigens. Immune responses to streptolysin O and/or deoxyribonuclease B were observed after 67% of the new GAS acquisitions. Persistently positive (>12 weeks) throat culture results were returned for 20% of the 41 subjects despite immune responses to homologous M peptides and/or shared antigens.

Conclusions. The availability of throat culture results, GAS isolates, and serial serum samples collected prospectively over a 2-year period of observation provided a unique opportunity for us to assess the serologic status of pediatric subjects before and after new pharyngeal acquisitions of GAS. With the exception of antibody responses to the homologous M peptides, no clear pattern of immune responses against the remaining GAS antigens was seen. There were no new immunologically significant acquisitions of *emm* types of GAS against which the subjects had preexisting elevated levels of antibodies against the homologous M peptide. The observation that 65% of new GAS acquisitions caused no symptoms yet were immunologically significant suggests that the majority of infections are not detected, which would result in missed opportunities for primary prevention of rheumatic fever and rheumatic heart disease with appropriate antimicrobial therapy.

Keywords. group A streptococcus; human immune responses; M protein; shared antigens.

Despite the fact that group A streptococcus (GAS) infections are among the most common infections in childhood and the global burden of disease is significant [1], relatively little is known about the human immune response after natural infection. The early work of Lancefield [2] emphasized the protective immunogenicity of M proteins in animal models. Prospective clinical studies were designed to measure the development of M antibodies and/or antibodies against common antigens, such as streptolysin O (SLO), deoxyribonuclease B (DNaseB), and C5a peptidase (SCPA), in patients with uncomplicated infection or acute rheumatic fever [3–5]. Likewise, most efforts to develop vaccines have centered on the M protein because of its ability to evoke protective immunity in animals [6] and the observation that immunity in humans seems to be type specific [7]. It has been suggested that protective immunity against GAS infection might be directed against both the M protein and broadly cross-reactive antigens shared by many or all M types [8]. A number of shared antigens have been identified using genomics, proteomics, reverse vaccinology, or more classical approaches that identify key virulence determinants [8, 9]. Many of the shared antigens have been shown to provide protection in

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animal models, and some were identified on the basis of the observation that human sera contain antigen-specific antibodies [9].

Longitudinal studies of the human immune response to GAS antigens, with the exception of M protein, SLO, DNaseB, and SCPA, after natural infection have not been performed thus far. In this study, we evaluated 195 serum samples obtained serially over a 24-month period from 41 pediatric subjects who experienced 51 new pharyngeal acquisitions of GAS [10, 11]. We determined the kinetics and antigen specificity of the immune responses by using a panel of 13 shared antigens and 18 type-specific M peptides.

MATERIALS AND METHODS

Study Subjects

Two previous studies designed to examine the possible association between GAS infection and pediatric autoimmune neuropsychiatric disorders associated with streptococci (PANDAS) enrolled the original 160 subjects [10, 11]. The subjects ranged in age from 6 to 15 years and were enrolled at multiple academic medical centers across the United States; the planned observation time was 108 weeks [12]. The current study evaluated a subset of 41 subjects who experienced 51 new pharyngeal acquisitions of GAS during the 2-year study period. A new immunologically significant acquisition was defined as a throat culture that tested positive for an *emm* type of GAS that had not been isolated previously and was followed by an immune response to 1 or more antigens in the enzyme-linked immunoassay (ELISA) panel. The study was approved by the institutional review boards of the University of Tennessee Health Science Center, the University of Minnesota, and the respective clinical sites that participated in the original studies.

Throat Cultures

Throat swabs were obtained on enrollment, every 4 weeks thereafter, and when the subjects showed any signs or symptoms of pharyngitis [10, 11]. Swabs were sent to the University of Minnesota Streptococcal Reference Laboratory, where group A β-hemolytic streptococci were identified, grouped, and *emm* typed [10, 11].

Serum Samples

Serum samples were obtained on enrollment and every 13 weeks from each study subject. In the intervals between scheduled collections, signs and symptoms of pharyngitis resulted in an additional serum collection and an additional convalescent sample 4 to 6 weeks after the acute episode [10, 11].

ELISA Antigens

Thirty-one GAS antigens, which included 18 M peptides and 13 conserved or semiconserved antigens, were used in these studies (Table 1). The M peptides selected were based on those M types recovered from the subjects in this study (M1, M2, M3, M4, M5, M6, M12, M18, M22, M28, M75, and M89) and additional M types frequently recovered from pharyngeal infections in the United States (M11, M49, M58, M77, M81, and M118) [13]. All of the M peptides have been shown to evoke bactericidal antibodies and were synthesized (GenScript, Piscataway, NJ) for a previous study [14]. The majority of the conserved or semiconserved antigens and the M peptides used in this study were selected on the basis of previous reports that indicated their immunogenicity and potential as vaccine components (Table 1). N-terminal peptides of the M-related proteins (Mrps) Mrp2 (83 amino acids [aa]), Mrp4 (93 aa), and Mrp49 (83 aa), which represent the 3 structural groups of MrpI, MrpII, and MrpIII, respectively, were cloned, expressed, and purified as described previously [15]. The J14 vaccine peptide, which copies in part a

Table 1. Group A streptococcus Antigens Evaluated

Antigen	Bacterium Location	*emm* Types in this Study Expressing the Antigen (% of Total Acquisitions)	Function	Reference(s)
Type-specific M peptides (1–6, 11, 12, 18, 22, 28, 49, 58, 75, 77, 81, 87, and 89)	Cell surface	All, type specific	Opsonic epitopes	31
Mrps, groups I, II, and III	Cell surface	2, 4, 22, 28, 75, 89 (39)	Opsonic epitopes	15
J14	Cell surface	All	Opsonic epitopes	6, 32, 33
SLO	Secreted	All	Hemolysin	12
DNaseB	Secreted	All	Degrades neutrophil nets	12
SCPA	Cell surface and secreted	All	Cleaves C5a	16, 34, 35
SpyCEP	Cell surface and secreted	All	Cleaves IL-8	17, 36
SSE	Secreted	1, 2, 3, 5, 6, 12, 18, 22, 75, 89 (86)	Tissue invasion	37
SOF	Cell surface and secreted	2, 4, 22, 28, 75, 89 (39)	Opsonic epitopes/fibronectin binding	18, 38
FBP54	Cell surface	All	Adhesin/fibronectin binding	19, 39
SpyAD	Cell surface	All	Cell division and adhesion	20
GAC	Cell Surface	All	Opsonic epitopes	40, 41

Abbreviations: DNaseB, deoxyribonuclease B ; FBP54, fibronectin-binding protein; GAC, group A carbohydrate; IL-8, interleukin 8; J14, C-repeat M peptide; Mrps, M-related peptides; SCPA, C5a peptidase; SLO, streptolysin O; SOF, serum opacity factor; SpyCEP, serine protease; SSE, serine esterase.

conserved C-terminal region of the M protein, was synthesized (Invitrogen, Carlsbad, CA) for a previous study [6]. Full-length SCPA was cloned and purified as described [16]. The serine protease SpyCEP [17] was cloned, expressed, and purified using polymerase chain reaction primers to amplify a 466-codon gene that encodes a truncated recombinant peptide. Streptococcus-secreted esterase (SSE) was cloned (Spy1718) and expressed from M15 GAS. Full-length serum opacity factor (SOF) from M4 streptococci and fibronectin-binding protein 54 (FBP54) were produced for previous studies [18, 19]. SpyAD [20] was cloned and purified as 2 recombinant peptides, one containing amino acids 38–242 of the mature protein and a second hybrid fusion protein containing amino acids 409–530, 581–605, and 742–801. Group A carbohydrate (GAC) was extracted from M28 GAS from our laboratory collection and purified using methods described previously [21]. SLO was purchased (Abcam, Cambridge, UK), and DNaseB was prepared by cloning and expressing the full-length DNaseB gene (*sdaB*) from M1 strain 90–226.

Control Sera and Establishing the Normal Range of Antibody Levels

Sera from 15 children aged 13 to 23 months from a previous study [15] were pooled and used as a negative control sample. All negative control serum samples diluted 1:200 resulted in an optical density at 405 nm (OD) below or equal to the background OD observed without primary antibody when screened against all shared antigens. Sera from 54 adults from a previous study [15] and from our laboratory collection were screened at a 1:200 dilution for antibodies against the shared antigens SpyAD, SOF, SSE, and FBP54. Three samples with high levels of antibodies (OD > 0.8; range, 0.825–1.540; mean, 1.13) against each of the 4 antigens were pooled and used as a positive control. For the remaining shared antigens (SCPA, SLO, DNaseB, J14, GAC, and SpyCEP), 83 samples from this study were chosen at random, diluted 1:200, and used to determine the range of antibody levels in the test sera. With the exception of SLO, SCPA, and DNaseB, a 1:200 dilution of serum was appropriate to yield an OD within the straight-line portion of the ELISA titration curve. When used at a dilution of 1:200, the OD values observed against SLO, SCPA, and DNaseB were at or above the upper limits of the straight-line regions of the ELISA curve. For these antigens, serum dilutions of 1:12 800 for SLO, 1:3200 for SCPA, and 1:3200 for DNaseB were required to yield OD values within the straight-line region of the ELISA curve to detect changes in antibody levels over time. Mrp-positive controls and baseline antibody levels were established from previously published studies [15].

ELISA

Nunc Maxisorp 96-well microtiter plates (Fisher Scientific, Waltham, MA) were coated with each protein antigen at a concentration of 0.5 μg per well and incubated for 1 hour at 37°C. GAC was conjugated to poly-L-lysine (Sigma-Aldrich, St. Louis, MO) using a previously published protocol [22] and was added at a concentration of 0.1 μg of carbohydrate per well. Serum samples were diluted as described above, and an ELISA was performed as reported previously [23].

Serum samples were analyzed in duplicate on separate occasions with 29 of the 31 antigens (Table 1). Pooled control sera for each antigen were run in duplicate, and the overall variation in OD from the mean value of 9 serum pairs was ~20% in all cases. In any given assay, if the results of paired experimental serum samples seemed disparate (≥25% difference in OD), the samples were reanalyzed. The GAC and J14 antibody assays were not performed in duplicate. However, samples for which changes in antibody levels against GAC and J14 from one time point to another were found, according to threshold values described below, were repeated for confirmation (18 samples).

Data Analysis and Statistics

An immune response to GAS antigens was defined by plotting sequential OD values on a standard ELISA curve created by titrating human MrpII antibodies [15]. A threshold for defining a change in antibody level from one time point to another was determined on the basis of the extrapolated position of the OD of the test sample on the standard curve. If the initial OD value was less than 0.250, an immune response to that antigen was defined as the equivalent of a 4-fold increase over the preceding value; the MrpII antibody dilutions were used as the reference. If the OD was within the straight-line portion of the curve, a 2-fold increase in antibody level from the previous value was chosen to represent an immune response to that antigen. Internal validation of the method was supported by the observation that in all but one instance, antibody responses that reached this threshold definition either continued to increase or were sustained in serum samples obtained at subsequent time points. The correlations among immune responses to SLO, DNaseB, SpyAD, FBP54, SSE, GAC, SpyCEP, and M peptides to each of the other antigens were assessed using the pairwise 2-tailed Fisher exact test.

RESULTS

Overview of Human Immune Responses to GAS Antigens

After the 51 new acquisitions of GAS observed, the immune responses were highly variable among subjects, even when different subjects acquired the same M type (Table 2). The average number of antigens to which a specific antibody response was detected after a new GAS acquisition was 3.5 (range, 1–8 antigens). With the exception of antibody responses to type-specific M peptides, there were no associations between the infecting M type and immune responses to the remainder of the GAS antigens tested. For example, M1 was responsible for 6 new acquisitions, and M6 was isolated from 5 subjects with a new

Table 2. Immune Responses to M Peptides and Shared Antigens After New Pharyngeal Acquisition of Group A streptococcus[a]

Patient No.	*emm*[b]	SLO	DNaseB	SCPA	SpyAD	FBP54	SSE	J14	GAC	SpyCEP	M Peptide	SOF	MrpI	MrpII	MrpIII	Symptoms	Antibiotic
13	1	–	+	+	–	–	+	–	–	+	+					–	–
672	1	–	+	–	–	–	–	–	–	–	–					+	–
678	1	–	–	–	+	–	+	–	–	–	+					+	–
760	1	–	–	+	+	–	–	–	+	–	+					–	+
768	1	–	–	–	–	–	+	–	–	–	+					+	–
784	1	+	+	+	-	+	–	–	–	–	+					+	+
3	2	–	+	–	+	–	–	–	–	–	–	+	–			+	+
14	2	–	–	–	+	–	–	–	–	–	+	–	+			–	–
19	2	–	–	–	+	–	–	–	–	–	+	+	+			–	+
17	3	+	–	+	-	–	–	–	–	–	–					+	+
19	3	–	–	–	–	–	+	–	–	–	+					–	–
25	3	–	+	–	–	–	+	–	–	–	–					–	–
26	3	+	+	–	+	–	–	–	–	–	–					–	+
626	3	+	–	–	–	–	+	–	–	–	+					–	–
752	3	+	–	+	+	–	–	–	+	–	–					+	+
4	4	+	–	–	–	–		–	–	–	–	–		+		–	–
11	4	+	+	+	+	–		–	–	–	+	+		+		–	–
6	5	–	–	–	+	–	–	–	–	–	+					–	+
12	5	+	+	+	+	–	–	–	–	–	+					–	–
785	5	+	+	–	+	–	+	–	+	–	+					+	+
5	6	–	–	+	+	–	–	–	–	–	+					–	–
679	6	–	–	+	–	–	–	–	–	+	–					–	+
760	6	–	–	+	+	–	–	–	–	–	+					+	+
763	6	–	+	–	–	–	+	–	+	–	+					–	–
768	6	+	–	–	–	–	+	–	+	–	+					–	–
14	12	–	+	–	+	–	–	–	–	–	+					–	–
27	12	+	+	–	–	–	+	–	–	–	+					–	–
29	12	–	+	–	+	–	-	–	–	+	+					–	–
626	12	–	–	–	–	–	+	–	–	–	+					–	–
627	12	+	+	+	–	–	–	–	–	–	–					–	–
745	12	–	+	–	–	–	–	–	–	–	–					+	+
790	12	+	+	+	–	–	–	–	–	–	–					–	–
21	18	+	+	+	–	–	–	–	–	–	+					+	–
762	18	–	–	–	+	+	–	–	+	–	–					+	–
763	18	–	–	–	+	+	–	–	–	–	+					–	–
781	18	–	–	–	+	–	+	–	–	–	+					–	+
8	22	–	+	–	+	–	–	–	–	–	+	+		+		–	–
9	28	–	–	–	+	–		–	–	+	+	+		+		–	–
20	28	+	–	+	+	+		–	–	+	+	–		–		+	–
23	28	–	–	+	-	-		–	–	–	–	–		–		+	–
24	28	–	–	–	+	-		–	–	–	+	+		–		+	–
743	28	+	+	–	-	+		–	+	+	+	+		+		–	–
16	75	+	+	+	–	–	–	–	–	–	–	+	–			–	–
25	75	+	–	–	–	–	+	–	–	–	+	–	–			–	–
517	75	+	+	+	+	–	–	–	–	–	–	+	+			–	–
550	75	-	+	–	–	–	–	–	–	–	–	–	–			+	+
763	75	+	+	+	–	–	–	–	–	+	–	+	+			–	–
768	75	+	+	–	+	–	+	–	+	–	–	+	–			–	–
2	89	+	–	–	–	–	–	–	–	–	+	+		–		+	+
19	89	–	+	–	–	–	–	–	–	–	+	–		–		–	–
567	89	+	+	–	+	–	–	+	–	–	–	–		–		+	+

Abbreviations: DNaseB, deoxyribonuclease B ; FBP54, fibronectin-binding protein 54; GAC, group A carbohydrate; J14, C-repeat M peptide; Mrp, M-related peptide; SCPA, C5a peptidase; SLO, streptolysin O; SOF, serum opacity factor; SpyCEP, serine protease; SSE, serine esterase.

[a] + or - indicates positive or negative antibody response to the antigen indicated. See Materials and Methods for the definition of a positive response. No entry in the table indicates that the *emm* type is not predicted to express the subject antigen.

[b] *emm* type of the GAS recovered from throat culture.

acquisition. Although 5 of the 6 subjects who acquired M1 GAS had subsequent immune responses to the M1 peptide, there was no consistent pattern of immune responses to the shared antigens tested (Table 2). Four of the 5 subjects who acquired M6 GAS mounted an immune response to the M6 peptide, but there was not a consistent pattern of antibody responses to the remainder of the antigens in the panel. Repeated pairwise Fisher exact tests were used to compare immune responses to SLO, DNaseB, SpyAD, FBP54, SSE, GAC, SpyCEP, and M peptides, but none of them revealed a statistically significant correlation.

Examples of the Kinetics and Antigen Specificity of Antibody Responses After Acquisition of GAS

After new acquisitions of GAS, various immune responses against multiple antigens were observed (Figures 1 and 2).

Immune Responses to Type-Specific M Peptides and Shared Antigens

Of the 51 new acquisitions of GAS observed, 32 (63%) resulted in increases in antibodies against the homologous M peptide. Examples of M peptide antibody responses and variable responses to shared antigens are shown in Figure 1. In the 32 subjects who mounted an immune response to the homologous M peptide (Table 2), 14 (44%) of 32 did not display a concomitant increase in antibodies against either SLO or DNaseB. In addition, 19 episodes of a new GAS acquisition revealed no M peptide immune response but were associated with antibody responses to an average of 3.3 shared antigens (range, 1–6), although not all GAS isolates were predicted to express all of the shared antigens (Table 2). Subject 567 (Figure 2A) mounted brisk antibody responses to SLO, DNaseB, and SpyAD but no response to the M89 peptide after a culture positive for M89 GAS.

Sequential Acquisition of More Than 1 M Type of GAS

Seven of the 41 subjects in this study experienced new pharyngeal acquisitions of more than 1 *emm* type of GAS (range, 2–3). In the majority of cases, the subjects mounted an antibody response against the homologous M peptide and variable antibody responses against shared antigens (Figure 2B–2D).

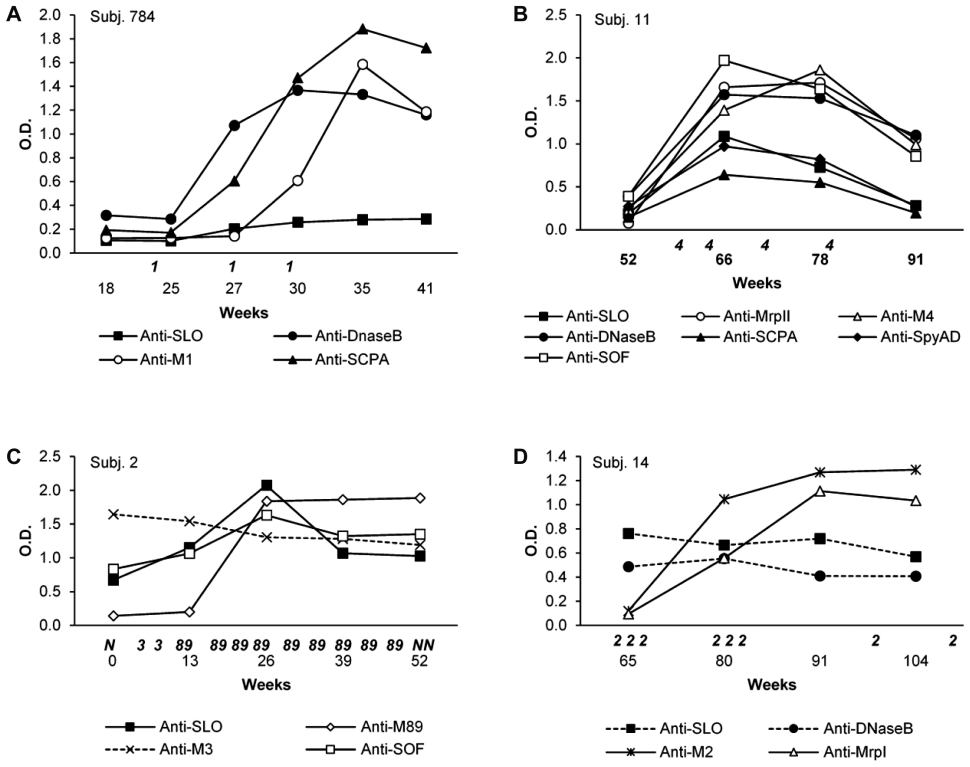

Figure 1. Examples of M peptide antibody responses and variable responses to shared group A streptococcus (GAS) antigens. The subject number is indicated for each set of data. Study weeks are represented on the horizontal axis. The numbers directly under the horizontal axis indicate the *emm* types of GAS recovered from throat cultures during the study, and N indicates a negative culture result. Dashed lines represent antigen-specific antibody levels that did not change during the observation period indicated. (A) Immune responses to the M1 peptide, deoxyribonuclease B (DNaseB), and C5a peptidase (SCPA) that were also sustained after the acquisition of M1 GAS were found in the serum from subject 784. The antibody response to streptolysin O (SLO) was much lower in magnitude but was sustained. (B) Subject 11 acquired a serum opacity factor (SOF)-positive M4 and mounted a brisk immune response to the M4 peptide, M-related peptide II (MrpII), SLO, DNaseB, SOF, SCPA, and SpyAD. (C) Subject 2 entered the study with a throat culture positive for M3 and elevated levels of antibodies against the M3 peptide. In week 13, the throat culture was positive for M89 GAS, and the subject responded with increases in antibody levels against the M89 peptide, SLO, and SOF. (D) No change in antibody levels against SLO or DNaseB in response to M2 GAS were found in the serum from subject 14, but immune responses to the M2 peptide and MrpI were mounted. Abbreviation: O.D., optical density.

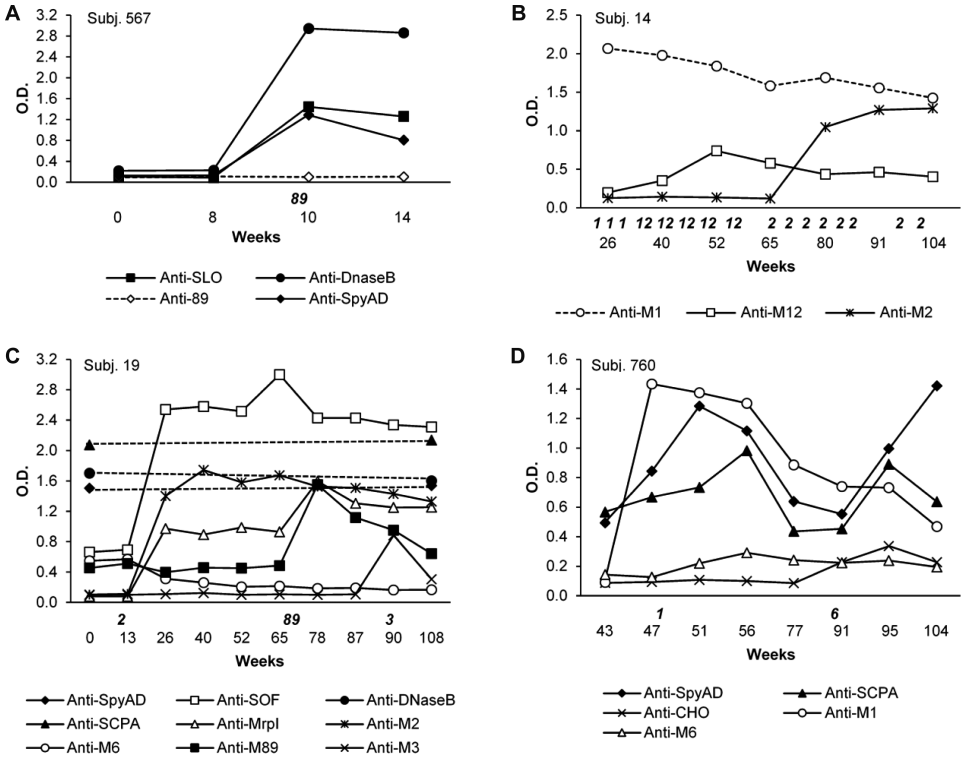

Figure 2. Examples of antigen-specific immune responses in 4 subjects after a new acquisition of group A streptococcus (GAS). The subject number is indicated for each set of data. Study weeks are represented on the horizontal axis. The numbers directly under the horizontal axis indicate the *emm* types of GAS recovered from throat cultures during the study. Dashed lines represent antigen-specific antibody levels that did not increase during the observation period indicated. (A) Subject 567 mounted brisk antibody responses to streptolysin O (SLO), deoxyribonuclease B (DNaseB), and SpyAD but no response to the M89 peptide after a culture positive for M89 GAS. (B) Subject 14 provides an example of sequential acquisitions of 3 different M serotypes of GAS over the course of the study. Throat cultures were positive on several occasions for M1 GAS, and the serum contained high levels of M1 peptide antibodies. By week 40, M1 was replaced by M12 GAS, and there was an immune response to the M12 peptide. At week 65, a throat culture was positive for M2 GAS, and there was an antibody response to the M2 peptide. (C) Subject 19 acquired 3 different M types (M2, M89, and M3) over the 2-year observation period. After each episode, there was an immune response to the homologous M peptide. The acquisition of M2 also triggered an antibody response against M-related peptide I (MrpI), and the acquisitions of serum opacity factor (SOF)-positive M2 and M89 were associated with increases in SOF antibodies. This subject also had persistently elevated levels of antibodies to DNaseB, C5a peptidase (SCPA), and SpyAD that did not change markedly from week 0 to 108. (D) An additional example of multiple GAS acquisitions was subject 760, who acquired 2 different *emm* types. A culture positive for M1 was associated with increases in antibodies against M1, SCPA, and SpyAD. Approximately 1 year later, a throat culture was positive for M6, which was associated with an increase in M6 peptide antibodies and more pronounced increases in antibody levels against SpyAD and SCPA. Abbreviations: CHO, group A streptococcus carbohydrate; O.D., optical density.

Immune Responses and Persistent Carriage of GAS

Of the 41 study participants, 8 (20%) were noted to have persistently positive throat culture results for the same strain for 12 weeks or longer (mean, 23.1 weeks; range, 12–52.8 weeks) despite immune responses to homologous M peptides and/or shared antigens. For example, subject 2 entered the study with a negative throat culture result, but then at weeks 6 and 9, the culture results were positive for M3 (Figure 1C). Because the positive M3 cultures occurred while M3 antibody levels were waning and did not trigger a significant immune response against other GAS antigens, it was not considered to be one of the 51 immunologically significant new acquisitions of GAS. Our assumption was that the detection of M3 represented persistent carriage after a previous infection before study entry and that the initial culture might have been falsely negative. The subject experienced symptoms of pharyngitis during week 12, and throat cultures were positive for M89 in week 13 and remained positive for M89 on 8 subsequent occasions despite the persistently elevated levels of M89 antibodies and SOF and SLO antibodies. Subject 14 had persistently positive culture results for M1 (38 weeks), M12 (26 weeks), and M2 (39 weeks), despite developing antibodies against homologous M peptides and shared antigens (Figure 2B).

Antibody Responses to SLO and DNaseB

Although there is not a standardized protocol for detecting antibodies against these antigens by ELISA, it was of interest to determine the predictive value of changes in antibody levels to SLO and DNaseB. Of the 51 new acquisitions of GAS, 34 elicited an increase in antibody levels against SLO and/or DNaseB (Figure 3A), which resulted in an overall sensitivity of 67% in detecting the new acquisition. When antibody responses to SCPA were included, the sensitivity increased to 76%. When antibody responses to SLO, DNaseB, SCPA, and any 1 additional

shared antigen were analyzed, the sensitivity increased to 98%. When only asymptomatic acquisitions of GAS were analyzed, similar predictive values were obtained (Figure 3B).

Type-Specific M Antibodies

Antibodies against the homologous M peptide were elicited after 63% of the GAS acquisitions (Table 2). Seven subjects acquired more than 1 M type during the study. None of the subjects experienced an immunologically significant new acquisition of a specific M type of GAS when there were elevated levels of preexisting serum antibodies against the homologous M peptide. Of the 18 M peptides tested, the average number against which the subjects had an elevated antibody level (OD > 0.7) at enrollment or developed after a new acquisition of GAS was 3.4 (range, 0–9).

Shared GAS Antigens

Some shared antigens, although common to many M types, are not predicted to be expressed by all GAS isolates recovered from subjects in this study (Table 1). On the basis of predicted antigen expression, immune responses were tabulated for each antigen, and the percentage of new acquisitions that elicited an immune response was determined (Figure 4). Overall, none of the shared antigens consistently evoked an immune response after a new GAS acquisition. The antigens that elicited the greatest number of immune responses were SOF (12 of 20 [60%]), DNaseB (26 of 51 [51%]), SpyAD (25 of 51 [49%]), and SLO (23 of 51 [45%]).

Immune Responses, Symptomatic Infections, and Antibiotic Therapy

As already stated, 65% of new GAS acquisitions caused no symptoms. The asymptomatic cases resulted in antibody responses to a mean of 3.7 of the antigens studied (range, 1–8),

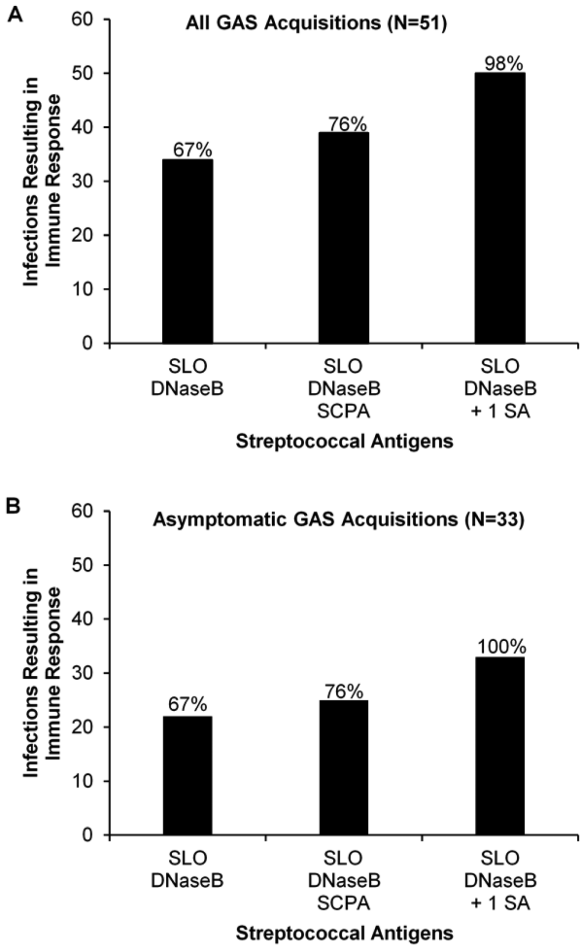

Figure 3. Immune responses to shared antigens after new pharyngeal acquisitions of group A *streptococcus* (GAS). Antibody responses to combinations of streptolysin O (SLO), deoxyribonuclease B (DNaseB), C5a peptidase (SCPA), or any 1 additional shared antigen were tabulated after all new GAS acquisitions (A) and asymptomatic acquisitions (B). Abbreviation: SA, streptococcal antigen.

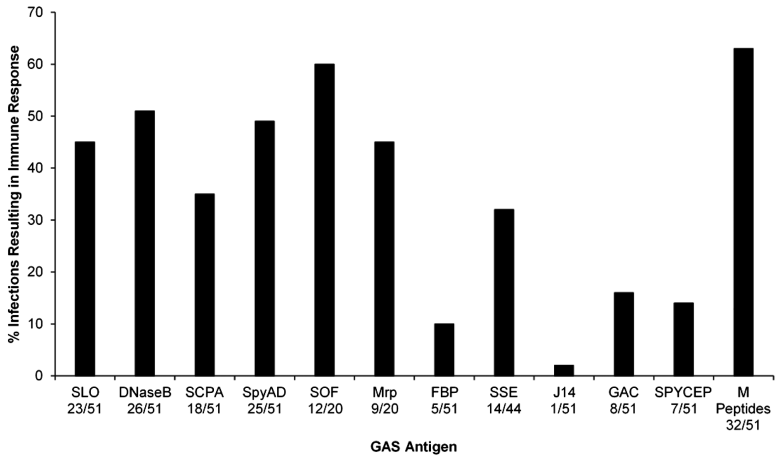

Figure 4. Antigen-specific immune responses in subjects with a new pharyngeal group A streptococcus (GAS) acquisition. Shown are the percentages of acquisitions that resulted in a specific antibody response, calculated using the number of GAS isolates predicted as the denominator to express each antigen (see Table 1). Abbreviations: DNaseB, deoxyribonuclease B; FBP, fibronectin-binding protein; GAC, group A carbohydrate; J14, C-repeat M peptide; Mrp, M-related peptide; SCPA, C5a peptidase; SLO, streptolysin O; SOF, serum opacity factor; SpyCEP, serine protease; SSE, serine esterase.

including the homologous M peptide. New acquisitions that did cause symptoms were associated with antibody responses to a mean of 3.0 antigens (range, 1–6). Antibiotic therapy was prescribed by the local treating physician in 16 (31%) of the 51 new acquisitions, after which there were antibody responses to a mean of 3.1 antigens (range, 1–6). When no antibiotics were prescribed (69% of cases), the subjects experienced antibody responses to a mean of 3.6 antigens (range, 1–8). Ten (56%) of the 18 subjects with a symptomatic acquisition received antibiotics, and 6 (18%) of the 33 asymptomatic subjects received antibiotics ($P = .01$, Fisher exact test).

DISCUSSION

The results of this study provided detailed information about the antigen specificity and kinetics of the human immune response after pharyngeal acquisition of GAS. The availability of throat culture results, GAS isolates, and serial serum samples collected prospectively during a 2-year study period of observation provided us a unique opportunity to assess the serologic status of pediatric subjects before and after a new pharyngeal acquisition of GAS. Although the subjects enrolled in this study were diagnosed with PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococci), to our knowledge, there is no reason to suspect that immune responses in this cohort would differ significantly from those of children in the general population. Previous studies evaluated the development of antibodies against M protein [3], SLO, DNaseB [12], and SCPA [5] in acute and convalescent serum samples after group A streptococcal infection. We believe that to date, this study is the most comprehensive prospective longitudinal analysis of immune responses to type-specific and shared antigens of GAS.

Previous studies examined the appearance of M antibodies after a new acquisition of GAS. However, all of those studies relied on the relatively insensitive bactericidal assay as an indicator of M antibodies [3, 24, 25]. Wannamaker et al [7] found an inverse correlation between the presence of serum bactericidal antibodies and symptomatic infection with the same M type of GAS, yet M antibodies did not correlate with transient acquisition of the homologous type. Quinn et al [25] studied 75 children with a new GAS acquisition and found that 88% did not have strain-specific bactericidal M antibodies before the acquisition. Only 30% of the subjects developed bactericidal antibodies within 6 weeks of the new GAS acquisition, and only one-third of those subjects showed concomitant antibody responses to SLO or hyaluronidase, which suggests that many of them might have been asymptomatically colonized or, alternatively, that the bactericidal assay was not sensitive enough to detect seroconversion. In our study, by using a sensitive and specific ELISA with type-specific synthetic M peptides, we found homologous M antibody responses in 63% of the new GAS acquisitions. No new immunologically significant acquisition of *emm* types of GAS against which the subjects had preexisting elevated levels of antibodies against the homologous M peptide were found. This result suggests an association between the presence of M antibodies and resistance to new pharyngeal acquisition of the homologous type of GAS, although we cannot state that with statistical significance. A more precise correlation will require a larger population-based longitudinal study that includes frequent serial cultures, comprehensive M peptide antibody assays, and the prevalence of specific M types circulating in the population during the study period.

Of considerable interest is our finding that 65% of the subjects with a new acquisition of GAS were asymptomatic, yet these subjects mounted immune responses against an average of 3.7 antigens. A previous study by Quinn et al [25] also found that 68% of their 75 subjects had asymptomatic new acquisitions of GAS. Altogether, the results indicate that most immunologically significant new acquisitions of GAS, similar to those of most pathogens, are asymptomatic. Many of these individuals continued to harbor the same organism for weeks or months. In some cases, the original M type was subsequently replaced by another M type, which might or might not have been associated with symptoms of pharyngitis. Our findings support the concept that there is a continuum of clinical scenarios associated with GAS in the pharynx that includes acute symptomatic pharyngitis, asymptomatic yet immunologically significant infection, persistent asymptomatic carriage of the same organism after infection, and asymptomatic acquisition and carriage of GAS without an immune response. The observation that such a high percentage of subjects experienced immunologically significant acquisitions of GAS and yet were asymptomatic underscores our current inability to discriminate between a harmless carriage state or colonization and actual infection. These results also indicate that the diagnosis and treatment of GAS pharyngitis, which is currently limited to those with symptomatic infection and who seek care, exclude the prevention of nonsuppurative complications in the majority of infected children, which is entirely consistent with previous observations that almost two-thirds of new cases of acute rheumatic fever are not preceded by symptomatic pharyngitis [26, 27].

In the clinical setting, evidence of recent streptococcal infection in the context of nonsuppurative complications such as acute rheumatic fever or glomerulonephritis is generally based on antibody levels against SLO or DNaseB [28]. These measurements represent one point in time, are not typically obtained from paired samples, and are often misleading [12]. In our study, we confirmed the finding by Johnson et al [12] that appropriate sequential samples define infection more accurately. In addition, we have shown that immunologically significant acquisitions of GAS, as evidenced by antibody responses to 1 or more shared antigens, occurred in the absence of increases in antibodies to SLO or DNaseB. These findings confirm that a larger antigen panel would increase the sensitivity of assays designed to detect recent GAS infection [29].

There is not an established immune correlate of protection against GAS infections in humans. One of our original hypotheses before undertaking these studies was that a predictable pattern of immune responses can guide the identification of shared, potentially cross-protective antigens for inclusion in vaccines. Our observation that the immune responses were quite variable might reflect the fact that a majority of new GAS acquisitions were not associated with symptoms that indicated a significant inflammatory response. Our findings do not preclude that an immune response to 1 or more of the shared antigens can be protective against subsequent infection, but the absence of a clear pattern does not particularly facilitate the identification of putative vaccine antigens. It is important to note that the majority of serum samples used in this study contained levels of antibodies against SLO, DNaseB, and SCPA that were much higher than those against the other antigens tested, which necessitated dilution of the serum samples because our primary goal was to detect increases in antibody levels in response to a new GAS acquisition. Contrary to the results of an earlier study [5], we did not consistently observe increases in anti-SCPA after the acquisition of GAS, which might be explained by technical differences in the assays or the fact that patients in the previous study were reported to have had pharyngitis [5]. The finding that all of the antigens studied were immunogenic in 1 or more subjects indicates that each has the potential to contribute to protection against infection. However, concluding that immune responses against any 1 of the antigens correlates with protection will likely require controlled vaccine trials with efficacy end points and detailed assessments of vaccine-induced immune responses [30].

Notes

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Potential conflicts of interest. J. B. D. is the inventor of certain technologies related to the development of group A streptococcal vaccines. The University of Tennessee Research Foundation has licensed these technologies to Vaxent, LLC, of which J. B. D. is the chief scientific officer and a member. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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