Quantitative and Qualitative Comparison of Virulence Traits, Including Murine Lethality, among Different M Types of Group A Streptococci

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Epidemiological studies have proposed an association between group A streptococci (GAS) bearing a particular M serological type and pathologic conditions such as streptococcal toxic shock syndrome (STSS). M1 and M3 GAS are isolated from STSS cases more frequently, whereas M4 and M12 GAS are isolated from non-STSS cases more frequently. To investigate whether there is any difference contributing the M-type association among GAS, we compared various virulence traits, including the murine lethality of M4, M12, M1, and M3 GAS clinical isolates, which are not clonally related to one another. Murine lethality, the activities of superantigens, streptolysin O, and nicotinamide adenine dinucleotide glycohydrolase, and the presence of the speA and speC genes were closely associated with M type. These results indicate that M types may serve, in part, as markers for strains/clones with particular profiles of virulence traits and mouse lethality.

Group A streptococci (GAS) cause a wide variety of infectious diseases, from relatively benign to life-threatening. GAS can be subtyped according to the serotypes of the M proteins expressed on the cell surface [1]. Particular M types of GAS have been associated with certain diseases [2], which suggests that there are differences in the expression of pathogenic traits among GAS with different M types. However, such M-type–associated differences among GAS have been neither fully investigated nor documented in an integrated manner.

M1 and M3 GAS have been found predominantly in patients with streptococcal toxic shock syndrome (STSS), a life-threatening GAS infection, in Japan, the United States, and Europe (http://idsc.nih.go.jp/pathogen/refer/str2000.pdf) [3–6]. Reports have been conflicting regarding the diversity of M types of GAS isolated from invasive infections [7–9]. However, a nationwide surveillance in Japan during 1992–2000 of >17,000 cases of M4, M12, M1, and M3 GAS infections indicated that the risk factor for STSS is ~10-fold higher in infections caused by M1 and M3 GAS than those caused by M4 and M12 GAS (table 1) (http://idsc.nih.go.jp/pathogen/refer/str2000.pdf). Therefore, we chose to study M4, M12, M1, and M3 GAS, to address the question of whether there are any M-type–associated differences among GAS.

The expression of a number of GAS pathogenic traits—including superantigenic toxins (SAGTs), strep-
that may arise from frequent passages in vitro, only fresh cultures from the same frozen stocks were used to repeat experiments. Bacterial suspensions were adjusted to the same cell density by measuring the optical density at 600 nm.

**Preparation of GAS isolates’ genomic DNA.** GAS isolates grown in 100 mL of BHI broth were suspended in 1 mL of 10 mM Tris-Cl (pH 7.8) that contained 1 mM EDTA and 1000 U of mutanolysin (Sigma-Aldrich) and then incubated for 1 h at 37°C. After the addition of 50 μg of lysozyme to the cell suspensions, they were incubated again for 1 h at 37°C. The pellets were frozen at −20°C and thawed in 3 cycles, then subjected to a standard procedure for genomic DNA preparation [13].

**emm typing of GAS isolates.** emm typing of GAS isolates was done according to the Centers for Disease Control and Prevention guidelines (http://www.cdc.gov/ncidod/biotech/strep/strepindex.html), with minor modifications. In brief, genomic DNA from GAS isolates was used as the template for polymerase chain reaction (PCR). The resulting PCR fragments were purified with a QIAquick PCR purification kit (QIAGEN) and sequenced with a Genetic Analyzer 310 system (Applied Biosystems Japan).

**Pulsed-field gel electrophoresis (PFGE) of GAS isolates.** GAS isolates from 10-mL cultures were suspended in 500 μL of 10 mM Tris-Cl (pH 8.0) that contained 1 mM NaCl (TN buffer). After adjusting the cell density to the same cell number, 150 μL of each cell suspension was mixed with 150 μL of 1.5% InCert agarose (FMC Bioproducts). The mixtures were added to 0.6 U of achromopeptidase and dispensed onto a paraffilm sheet, to make solid plugs. The resulting plugs were transferred into tubes, and 1 mL of TN buffer was added, followed by incubation for 1 h at 50°C. The solution was replaced with 1 mL of 6 mM Tris-Cl (pH 8.0) that contained 50 μg of RNase, 1 mg of lysozyme, 50 U of mutanolysin, 1 M NaCl, 50 mM EDTA, 0.2% sodium deoxycholate, 0.5% sodium laurylsarcosine, and 0.5% Brij-58 and incubated for 1 h at 37°C. Finally, the solution was changed to 1 mL of a buffer that contained 0.5 M EDTA (pH 9.0), 0.1% sodium laurylsarcosine, and 1 mg/mL of proteinase K and incubated overnight at 50°C. After washing with the TN buffer, the plugs were treated with Smal for macrorestriction and applied to a GenePath system (Bio-Rad Laboratories). PFGE was done for 24 h at 6 V/cm with linear ramping.

**Determination of LD₅₀ values of GAS isolates.** The GAS isolate suspensions in PBS (0.5 mL) were injected intraperitoneally at several dilutions into female 6–7-week-old ddY mice (Sankyo Lab Service) (7 mice/group, 4 or 5 groups for different GAS isolates). Exact numbers of colony-forming units of the injected bacteria were determined by incubating each GAS sample on sheep blood-agar plates in duplicate at adequate dilutions. The data were analyzed for significance according to the Probit method [14], to determine LD₅₀ values for a 7-day period, with

<table>
<thead>
<tr>
<th>M type (T type)</th>
<th>Cases of STSS</th>
<th>Total no. of cases</th>
<th>STSS cases as % of total no.</th>
<th>STSS risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4 (T4)</td>
<td>2</td>
<td>5159</td>
<td>0.039</td>
<td>1.00</td>
</tr>
<tr>
<td>M12 (T12)</td>
<td>6</td>
<td>6703</td>
<td>0.090</td>
<td>2.31</td>
</tr>
<tr>
<td>M1 (T1)</td>
<td>32</td>
<td>4800</td>
<td>0.667</td>
<td>17.20</td>
</tr>
<tr>
<td>M3 (T3)</td>
<td>10</td>
<td>1258</td>
<td>0.795</td>
<td>20.50</td>
</tr>
</tbody>
</table>

**NOTE.** No. of cases of STSS and all GAS infections for each M type, taken from a nationwide surveillance of GAS infection in Japan, 1992–2000 (http://idsc.nih.go.jp/pathogen/refer/str2000.pdf), were used for the calculations. The percentage of cases of STSS among the total no. of cases of each GAS M type was normalized for M4 GAS and presented as the risk factor for STSS.

* As based on T-type analysis, because the no. of cases as based on M-type analysis was not available. A correlation between T type and M type has been reported elsewhere [44].
95% confidence intervals. The analyses were done using a Microsoft Excel macro written by Dr. S. Okumura (http://rd.vector.co.jp/vpack/browse/person/an019638.html). The LD₅₀ values obtained were transformed into logarithmic form and used for the calculation to obtain the geometric mean of LD₅₀ values for each M type and for statistical analysis. All animal experiments were done according to the guidelines of the Ethics Review Committee of Animal Experiments of Tokyo Women’s Medical University.

**PCR analysis of the speA, speB, and speC genes.** To detect speA, speB, and speC simultaneously, a PCR-based method designed by Inagaki and Watanabe [15] was used, with minor modifications. In brief, 25 μL of PCR mixtures in Taq buffer that contained 5 U of Taq (Takara), 0.2 μL of each of the primers indicated in table 2, and genomic DNA of GAS isolates as templates. PCR amplification was done with a PC-700 thermocycler (ASTEC) using the following parameters: 1 cycle of incubation at 95°C for 1 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and 1 extension cycle at 72°C for 3 min. To confirm amplification of the genes, agarose gel electrophoreses were performed.

**Determination of SPE-A, SPE-B, and SPE-C.** SPE-A, SPE-B, and SPE-C in the culture supernatants of GAS isolates were determined by a quantitative Western blotting method that used antibodies to SPE-A, SPE-B, or SPE-C, as described elsewhere [16]. In brief, culture supernatants of GAS isolates (2 μL/lane for SPE-B and SPE-C or 6 μL/lane for SPE-A) were run on the same blot with serially diluted standard SPE-A and SPE-C (Toxin Technology), 0.625–2.5 ng/lane, or SPE-B (Toxin Technology), 12.5–50 ng/lane. Each protein was detected by serial treatment of the blots with polyclonal anti–SPE-A, –SPE-C (a gift from Dr. Yuko Nemoto, Iwate Medical University, Morioka, Japan), or –SPE-B (Toxin Technology), horseradish peroxidase–conjugated anti-rabbit IgG, and Western blotting chemiluminescence luminol reagent (Santa Cruz Biototechnology). Developed films were scanned to digitize the data, and each band was quantified by using the National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/index.html) on the basis of the standard curves of SPE-A, SPE-B, and SPE-C. For SPE-B, the active form migrating at 28 kDa was quantified separately from the 40-kDa precursor protein.

**Determination of the T cell–stimulating activities of culture supernatants.** To quantify the T cell–stimulating activities of the culture supernatants of GAS isolates, we measured the activity that induced interleukin (IL)–2 production from human T cells [12]. In brief, human peripheral blood mononuclear cells (PBMCs; 2 × 10⁵/well) were cultured in 48-well plates in RPMI 1640 medium that contained 10% fetal calf serum, 50 μL of 2-ME, and 100 U/mL of penicillin and streptomycin, and stimulated with 5000-fold diluted culture supernatants of GAS isolates for 24 h. This dilution rate was chosen on the basis of preliminary experiments to obtain a linear response of IL-2 production. IL-2 activity in the culture fluid was determined with a bioassay that used IL-2–dependent CTLL-2 cells.

**Determination of SLO activity.** GAS isolates were grown overnight in BHI broth that contained 28 μm trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butan (E-64; Sigma-Aldrich), an inhibitor of cysteine proteinase. The cultures were frozen at −80°C, thawed, and centrifuged at 12,000 g to obtain the supernatants. Serially diluted culture supernatants (100 μL) in PBS that contained containing 0.5% bovine serum albumin, 0.0005% trypan blue, and 25 mM 2-ME were incubated for 10 min at room temperature. After incubation, 100 μL of 5% sheep red blood cell (SRBC) solution was added and incubated for 1 h at 37°C. After centrifugation at 500 g to remove nonlysed SRBCs, the supernatants were measured for absorbance at 540 nm to determine the ratio of SRBC lysis. One hemolysis unit was defined as the hemolysin activity yielding 50% of maximum SRBC lysis.

**Determination of hyaluronic acid content.** The amount of hyaluronic acid was quantitated as described elsewhere [17]. In brief, bacteria cultured in 10 mL of BHI broth were washed twice with water. After adjusting the cell density to be the same as the optical density, 200 μL of the cell suspension was added to 400 μL of chloroform and shaken vigorously, to convert hyaluronic acid to the aqueous phase. After centrifugation at 12,000 g, 25-μL samples were added to 100 μL of Stains-all solution that contained 0.02% Stains-all (Sigma-Aldrich), 0.06% glacial acetic acid, and 50% formamide, and the mixtures were measured for absorbance at 640 nm. The absorbance was compared with a standard curve obtained by human umbilical hyaluronic acid (Sigma-Aldrich).

**Determination of NAD glycohydrolase activity.** NAD glycohydrolase activity was determined using an indirect spectrophotometric assay described elsewhere [18]. In brief, the culture supernatants of GAS isolates were combined with 0.666 M potassium phosphate buffer (pH 7.35) and 1.25 μM NAD and incubated for 20 min at 37°C. After the addition of 0.6 M trichloroacetic acid (TCA), the samples were centrifuged at 12,000 g, and the supernatants were added to a reaction buffer that contained 70 mM glycine, 16.4 mM sodium pyrophosphate, and

**Table 2.** Polymerase chain reaction primers used to detect speA, speB, and speC in group A streptococcal isolates.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primer</th>
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<tr>
<td>SPE-A1</td>
<td>GCTCAACAAGACCCCCGATCC</td>
</tr>
<tr>
<td>SPE-A2</td>
<td>T6ATAGGCTTGGATACCATCG</td>
</tr>
<tr>
<td>SPE-B1</td>
<td>GATCAAACCTTGCGTAACCG</td>
</tr>
<tr>
<td>SPE-B2</td>
<td>AGGTGGATGCGTACAACACGC</td>
</tr>
<tr>
<td>SPE-C1</td>
<td>GACTCTAAAGAAGACATTGC</td>
</tr>
<tr>
<td>SPE-C2</td>
<td>AGTCCTCTCATTGGTGAGTC</td>
</tr>
</tbody>
</table>
Figure 1. LD₅₀ values, in mice, of group A streptococci (GAS) isolates of different M types and from streptococcal toxic shock syndrome (STSS) or non-STSS infections. M4, M12, M1, and M3 GAS isolates from non-STSS (white squares) or STSS (black squares) infections were injected into mice intraperitoneally (7 mice/group, 4 or 5 different densities), and mortality was observed for 7 days. LD₅₀ values were calculated on the basis of the Probit method [14] and are presented with 95% confidence intervals. Horizontal dotted lines denote geometrical mean LD₅₀ values in each GAS group. Results of the Mann-Whitney U test of geometrical mean LD₅₀ values among M types are presented in the inset table.

10 mM semicarbazide. To initiate the reducing reaction of NAD to NADH, 0.55 mg/mL of alcohol dehydrogenase was added, and the sample was incubated for 10 min at room temperature. The absorbance of the samples at 340 nm was compared with that of the control sample to which TCA had been added before NAD.

**Statistical analysis.** The data were analyzed for significance by the Mann-Whitney U test for nonparametric data. The analyses were done by using a worksheet for MS-Excel, Stat-macros, written by Dr. Tadao Hirota (International Christian University, Tokyo, Japan; http://www.tuat.ac.jp/~ethology/Columbo/Stat/index.html). P<.05 was considered to be statistically significant.

**RESULTS**

**Profiles of M4, M12, M1, and M3 GAS clinical isolates.** For the present study, we investigated the pathogenic traits of M4, M12, M1, and M3 GAS clinical isolates (table 3). Several reports have suggested that GAS clinical isolates sharing the same M type often show clonal characteristics, even if they are isolated from different pathologic incidences [16, 19, 20]. To generalize our results on M type, we attempted to increase the heterogeneity of the bacteria subjected. Thus, we chose GAS isolates that differed among the pathologic conditions of patients, years obtained, and geographical regions of isolation and in terms of the PFGE patterns within the same M-type isolates. Eight M4 isolates were divided into 5 PFGE patterns, 8 M12 and 8 M1 isolates were divided into 4 PFGE patterns, and 11 M3 isolates were divided into 2 PFGE patterns consisting of 10 in one and 1 in the other.

**Determination of LD₅₀ values of GAS isolates.** We have reported elsewhere that GAS strains isolated from STSS infections showed higher lethality in mice than those from non-STSS infections [12]. To investigate the difference in murine lethality with regard to M type, we determined the LD₅₀ values of M4, M12, M1, and M3 isolates. GAS isolates were injected intraperitoneally into mice, and lethality was determined by observation for 7 days (figure 1, table 3). The M type of each GAS isolate demonstrated a characteristic LD₅₀ pattern. The mean LD₅₀ values of M4 isolates (1.0 x 10⁷ cfu/mouse) was significantly higher than those of M12 (2.1 x 10⁵ cfu/mouse; P = .0013) and M1 (6.3 x 10⁶ cfu/mouse; P = .0003) isolates. As was mentioned above, the mean LD₅₀ values of M12 isolates was significantly higher than that of M1 isolates (P = .0032). The LD₅₀ values of M3 isolates were very heterogeneous (range, 8 x 10⁻⁶–2 x 10⁴ cfu/mouse), although the mean value of M3 isolates (9.4 x 10⁵ cfu/mouse) was significantly lower than that of M4 isolates (P = .0093). Three of 11 M3 isolates (M3-d–f) had very low LD₅₀ values, <5 x 10⁵ cfu/mouse, indicating that a certain percentage of M3 isolates is highly virulent.

**Distribution of speA and speC genes and protein expressions in GAS isolates.** SAGTs, which exert a toxic effect in a T cell–dependent manner [21–23], have been implicated as the major causative factor in the pathogenesis of GAS. We analyzed the gene distribution of major streptococcal SAGTs, speA and speC, by PCR and their expression in GAS isolates by quantitative Western blotting (figure 2).

In PCR analysis, speA was amplified from all M1 and M3 isolates but not from all M4 and M12 isolates. speC was positive in all M4 and M12 isolates and in 5 of 8 M1 isolates (M1-1–4 and M1-b). In contrast, speC was negative in all M3 isolates and in 3 of 8 M1 isolates (M1-a, M1-c, and M1-d). These
Table 3. Epidemiology and pulsed-field gel electrophoresis (PFGE) banding patterns of group A streptococcal isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Location</th>
<th>Year of isolation</th>
<th>Disease</th>
<th>T type</th>
<th>emm type</th>
<th>PFGE profilea</th>
<th>LD50 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4-1</td>
<td>Fukusima</td>
<td>1994</td>
<td>Pharyngitis</td>
<td>4</td>
<td>4</td>
<td>4d</td>
<td>3.4 × 10^6</td>
</tr>
<tr>
<td>M4-2</td>
<td>Kagoshima</td>
<td>1995</td>
<td>Pharyngitis</td>
<td>4</td>
<td>4c</td>
<td>2.1 × 10^6</td>
<td></td>
</tr>
<tr>
<td>M4-3</td>
<td>Kagoshima</td>
<td>1995</td>
<td>Pharyngitis</td>
<td>4</td>
<td>4b</td>
<td>2.0 × 10^6</td>
<td></td>
</tr>
<tr>
<td>M4-4</td>
<td>Chiba</td>
<td>1994</td>
<td>Pharyngitis</td>
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<td>4a</td>
<td>1.3 × 10^6</td>
<td></td>
</tr>
<tr>
<td>M4-a</td>
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<td>1996</td>
<td>STSS</td>
<td>4</td>
<td>4e</td>
<td>8.1 × 10^6</td>
<td></td>
</tr>
<tr>
<td>M4-b</td>
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<td>1993</td>
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</tr>
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</tr>
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</table>

NOTE. STSS, streptococcal toxic shock syndrome.

a Banding patterns were named arbitrarily. PFGE profiles of different emm types were always different.
b Isolated from a patient with STSS during delivery.

results were in accordance with the analyses of SPE-A and SPE-C proteins (see below) and indicate that PCR amplification for each gene in the PCR system was highly specific.

To quantify the SPE-A and SPE-C proteins specifically, we developed a quantitative Western blotting system by modification of a similar system applied for SPE-B quantification [16] (figure 3). Culture supernatants of M4 isolates, all of which were negative for speA in PCR analysis (figure 2), did not reveal the presence of the SPE-A protein. Likewise, Western blots of culture supernatants of M3 isolates, all of which were negative for speC in PCR analysis, did not detect the presence of the SPE-C protein. Together, these results indicate that the antibodies used in the present study were highly specific. Quantification by Western blotting and the inclusion of known standards in each blot generated reproducible results (figure 2). SPE-A was quantitatively detected in all M1 and M3 isolates.
The amount of SPE-A was very high in only 1 of 8 M3 isolates (M3-f) and was moderate in the other 7 isolates. It was also moderate in 5 of 8 M1 isolates and was low in the other 3 isolates. SPE-C was detected in 7 of 8 M4 isolates, 7 of 8 M12 isolates, and 5 of 8 M1 isolates. The amount of SPE-C was high to moderate in 4 of 8 M4 isolates and in 5 of 8 M12 isolates and was low or undetectable in the other isolates. The mean difference of the SPE-C production levels between M4 and M12 isolates ($P = .506$) and the mean difference of SPE-A production levels between M1 and M3 isolates ($P = .195$) were not statistically significant.

**Measurement of whole SAGT activity produced by GAS isolates.** It has been shown that GAS produces many SAGTs in addition to SPE-A and SPE-C [22, 24]. The strength of the
Figure 4. Quantification of superantigenic toxin (SAGT) activity produced by group A streptococci (GAS) isolates. Whole SAGT activity produced by GAS isolates from non–streptococcal toxic shock syndrome (STSS) (white bars) or STSS (black bars) infections was quantified by analyses of interleukin (IL)–2 production that used peripheral blood mononuclear cells stimulated with the culture supernatants, as described in Materials and Methods. Data are averages of 3 independent cultures and are expressed as means ± SE. Horizontal lines denote mean values in each GAS group. Results of the Mann-Whitney U test of mean LD₅₀ values among M types are presented in the inset table.

T cell–stimulating activity, a major biological trait of SAGTs, may not be equal among distinct SAGTs. Therefore, it is important to analyze the respective capability, as reflected by the amount of T cell–stimulating activity produced, of GAS isolates to produce SAGT activity as a whole. As shown in figure 4, we examined the activities of culture supernatants of GAS isolates to induce IL-2 production by PBMCs. The level of IL-2 production induced by the GAS isolates showed marked association with their M types. The mean levels of IL-2 production induced in M4 and M12 isolates (16.5 ± 2.3 and 12.5 ± 0.9 U/mL, respectively) were significantly higher than those in M1 and M3 isolates (6.1 ± 1.4 and 2.6 ± 0.4 U/mL, respectively) (P < .01 for each combination). Of interest, in M1 isolates, all speC-positive isolates showed significantly higher induction of IL-2 production than did speC-negative isolates. These results were reproducible in analyses that used PBMCs from different donors, which indicates that the higher production of IL-2 by PBMCs stimulated with culture supernatants from M4 and M12 isolates was not due to prior exposure of the donor to M4 or M12 GAS or to individual donor differences.

SPE-B production by GAS isolates. SPE-B, a cysteine proteinase, has been implicated in the pathogenesis of tissue damage in GAS infections [24]. As shown in figure 5, we compared the production of SPE-B by GAS isolates by quantitative West-
M-Type–Related Differences among GAS

Figure 6. Hemolysin production levels of group A streptococci (GAS) isolates. Streptolysin O (SLO) activity in the culture supernatants of GAS isolates from non–streptococcal toxic shock syndrome (STSS) (white bars) or STSS (black bars) infections were quantified as described in Materials and Methods. Data are averages of 3 independent cultures and expressed as means ± SE. Statistical analysis results of mean LD₅₀ values among M types are presented in the inset table.

Figure 7. NAD glycohydrolase activity levels produced by group A streptococci (GAS) isolates. NAD glycohydrolase activity in the culture supernatants of GAS isolates from non–streptococcal toxic shock syndrome (STSS) (white bars) or STSS (black bars) infections was quantified by the NAD degradation activity of GAS culture supernatants, as described in Materials and Methods. Data are averages of 3 independent cultures and expressed as mean ± SE. Results of the Mann-Whitney U test of mean LD₅₀ values among M types are presented in the inset table.

ern blotting [16]. Although analysis using the conventional PCR method revealed that all examined isolates carried speB (data not shown), the SPE-B protein was detected in few GAS isolates. In M4 isolates, the amount of SPE-B was high in 3 isolates from non-STSS infections and low or undetectable in the other isolates. In the M12 isolates, SPE-B was low or undetectable. In M1 and M3 isolates, it was high to moderate in 2 isolates each from non-STSS infections and low or undetectable in the other isolates. The mean difference of the SPE-B production levels among M4, M12, M1, and M3 isolates was not statistically significant for any combination of M types.

Production of SLO by GAS isolates. SLO, which exerts a lethal effect on experimental animals via a direct toxic effect on target cells, has been implicated in the pathogenesis of GAS [25]. As shown in figure 6, we analyzed the production of SLO by GAS isolates. We found SLO activity to be inversely related to the amount of SPE-B in the culture supernatants of various GAS isolates (data not shown), which indicates that SPE-B, as has been suggested elsewhere [26, 27], degrades SLO. Preliminary experiments showed that the addition of an inhibitor of cysteine proteinase, E-64, to the cultures of SPE-B–producing GAS isolates significantly increased SLO activity. Therefore, we examined the production of SLO by GAS isolates in the presence of E-64. The growth rate of GAS isolates and the activity of the other traits examined in the present study were not affected by addition of E-64 (data not shown). The level of SLO activity produced
Figure 8. Hyaluronic acid capsule quantification of group A streptococci (GAS) isolates. Hyaluronic acid contents of GAS isolates from non–streptococcal toxic shock syndrome (STSS) (white bars) or STSS (black bars) infections were quantified as described in Materials and Methods. Data are averages of 2 independent cultures and expressed as means ± SE. Results of the Mann-Whitney U test of mean LD_{50} values among M types are presented in the inset table.

by GAS isolates was associated with M type. The mean SLO activity in M4 isolates (25.7 ± 5.8 U/mL) was significantly lower than those of M1 (64.7 ± 12.9 U/mL; P = .0106) and M3 (62.0 ± 5.6 U/mL; P = .0003) isolates. Similarly, the mean SLO activity in M12 isolates (38.0 ± 6.2 U/mL) was significantly lower than that of M3 isolates (P = .0073).

**NAD glycohydrolase activity.** It has been reported that M1 GAS strains isolated after 1988 were positive for production of NAD glycohydrolase, which may contribute to GAS virulence by causing cytotoxicity to host cells [18, 28]. As shown in figure 7, the production of NAD glycohydrolase was quantified by measuring the enzymatic activity in culture supernatants of M4, M12, and M3 GAS isolates, in addition to M1 isolates that had mostly been isolated after 1988. The level of NAD glycohydrolase activity by GAS isolates was associated with their M type. M4 (1.54 ± 0.18 U/mL) and M3 (1.63 ± 0.06 U/mL) isolates produced significantly higher amounts of glycohydrolase activity than M12 (0.71 ± 0.11 U/mL; P = .0106 and P = .0008, respectively) and M1 (1.17 ± 0.09 U/mL; P = .0106 and P = .0013, respectively) isolates. The difference between the mean activity of M12 isolates and M1 isolates was also statistically significant (P = .005).

**Hyaluronic acid capsule production.** The hyaluronic acid capsule reportedly confers resistance to phagocytosis and enhances the virulence of GAS [29–31]. As shown in figure 8, the production of the hyaluronic acid capsule was examined by the quantification of hyaluronic acid in GAS isolates. The hyaluronic acid content varied among the 4 M types of isolates, and there was no statistically significant difference associated with their M types.

**DISCUSSION**

In the present study, we determined murine LD_{50} values and compared the major GAS virulence traits of M4, M12, M1, and M3 GAS clinical isolates, to explore whether the features of these traits, such as the level of production, could be associated with the M type. We found several associations of these traits with their M types (table 4). Because the isolates we used are not considered to be clonally related (table 3), we conclude that the pathologic traits of GAS isolates—such as LD_{50} values (except for M3), the presence of speA and speC, and activities of SAGTs, SLO, and NAD glycohydrolase—are directly related, by unknown mechanisms, to their M types.

In the mouse lethality analysis of GAS isolates, the order of mean LD_{50} values of M4, M12, and M1 isolates was M4 > M12 > M1. Although the LD_{50} values of M3 isolates were highly variable and might not be suitable for statistical comparison, ~30% of M3 isolates demonstrated lower LD_{50} values than the mean LD_{50} values of M1 GAS isolates, which suggests that a certain percentage of M3 GAS isolates is as highly virulent as M1 isolates. We found a similarity between the order of risk factors for STSS of M4, M12, M1, and M3 GAS isolates, as based on epidemiological data and the observed mouse lethality of M4, M12, M1, and M3 isolates. The order of STSS risk factor among M types GAS in Japan was M4 > M12 > M1 ≈ M3 (table 1). Such an accordance suggests that the GAS differences closely associated with M type actually underlie the mechanism of the M-type bias of STSS in humans. Our results also suggest that the experimental animal model used in our study reflects, at least in part, the pathogenic mechanism of STSS in humans.
Thus, our results provide a basis for comparing GAS virulence to causes of STSS in vivo by improving the model based on the accordance with epidemiological studies.

In the present study, we detected speA in all of the M1 and M3 isolates. Several investigators have reported that speA is predominantly harbored in M1 and M3 GAS [8, 32–36]. Conversely, speC is predominantly harbored in M4 and M12 GAS [35]. Our data are in agreement with those reports. However, it is assumed that speA and speC are transferred among GAS by phages, which suggests that the gene distribution observed in the present study may be tentative. Indeed, a recent analysis of GAS clinical isolates—including M4, M12, M1, and M3 types—has indicated that there is no bias among speA and speC distribution in those M types [9]. This observation may suggest the beginning of the spreading of the genes among GAS.

Although SAGTs have been implicated in the pathogenic mechanism of GAS [22, 37], the production level of the whole SAGT activity of GAS has not been well characterized except in our previous study [12] and a study that analyzed M1 isolates [38]. In the present study, we found the levels of SAGT produced by GAS to be closely associated with their M type. Although the whole SAGT activity of GAS isolates showed marked association with M type, levels of SPE-A and SPE-C production varied among the isolates (figure 3), as has been shown in M1T1 isolates [37]. We conducted inhibition experiments using anti-SPE-C antibody in a mitogenic activity assay of GAS culture supernatants on PBMCs. As was expected from the level of SPE-C production in each isolate, the inhibition ratio against whole SAGT activity varied widely, ranging from 10% to 80% among isolates (data not shown). These data indicate that the molecular species and the amount of each SAGT produced by GAS varies widely among GAS isolates, regardless of M type. Although we did not address the question of which SAGT mainly contributes to the rest of the SAGT activity in the culture supernatant, streptococcal mitogenic exotoxin Z (SMEZ) is a feasible candidate, because SMEZ shows 10 times higher activity than SPE-A [39]. Taken together, these observations indicate that the quantification of whole SAGT activity produced by GAS may be very informative for evaluating the role of SAGT in the pathogenic mechanism of GAS in comparison with the quantification of each SAGT separately, as we reported elsewhere [12].

SLO has been regarded to be an important virulence trait of GAS. Recently, deletion mutants of SLO were successfully established and used for animal experiments that implicated SLO in the pathogenesis of GAS [25]. We found the SLO production level of GAS to be associated with their M types. Because M3 and M1 GAS, which are high-risk GAS for STSS, produce higher levels of SLO than M4 and M12, SLO production levels may be one of the critical factors for STSS pathogenesis.

In our study, SPE-B was detected in 17 of 32 isolates, although all of them were positive for the speB gene. Similar varied expression of SPE-B in M1T1 isolates has been reported elsewhere [38]. Thus, our data support the existence of an on-off regulatory mechanism(s) for SPE-B expression and a posttranslational regulatory mechanism controlling its activity, as has been suggested elsewhere [16]. We did not observe any association between SPE-B production levels and M type. It has been reported recently that SPE-B expression in M1T1 isolates is inversely related to disease severity, as based on the active form of 28-kDa SPE-B being quantified specifically [16]. We did not find high–SPE-B–producing GAS in STSS isolates of M4, M1, or M3 GAS. Thus, the inverse relation between SPE-B production and the disease severity of GAS may be extended to other M types of GAS in addition to M1 GAS.

It has been reported that M1 GAS strains isolated after 1988 were positive for the production of NAD glycohydrolase, whereas virtually all strains isolated before 1988 were negative, which suggests the possible association of the enzyme activity with severe GAS infection [18]. We have confirmed the NAD glycohydrolase activity production of M4, M12, M1, and M3 isolates reported elsewhere [40]. The NAD glycohydrolase activity of GAS isolates was associated with their M type, although there was a discrepancy in the rank order of production levels between our data and those elsewhere. This discrepancy may be due to differences in the GAS culture conditions used in the 2 studies. In our study, there was no difference in NAD glycohydrolase activity between M1 isolates from STSS and non-STSS samples, as was the case in a recent investigation in Australia [41]. These observations suggest that the transition of M1 GAS to a NAD glycohydrolase–active type is apparently almost complete.

The hyaluronic acid capsule of GAS has been suggested to interfere with host defense mechanisms, including phagocyte function, antibodies, and complement [24, 42]. Our results indicate that the hyaluronic acid capsule levels vary among isolates, showing no correlation with M type. It also has been

Table 4. Association of the gene distribution of speA and speC and expression of superantigenic toxins (SAGTs), streptolysin O (SLO), and NAD glycohydrolase with M type.

<table>
<thead>
<tr>
<th>Expressed factor</th>
<th>M4</th>
<th>M12</th>
<th>M1</th>
<th>M3</th>
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<td>Low</td>
<td>Variable</td>
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<td>speA and speC</td>
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<tr>
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<td>Variable</td>
<td>Variable</td>
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</tr>
<tr>
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<td>Low</td>
<td>Low</td>
</tr>
<tr>
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<td>Variable</td>
<td>Variable</td>
<td>Low</td>
</tr>
<tr>
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<td>Low</td>
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<td>High</td>
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<td>Mid</td>
<td>High</td>
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<td>Hyaluronic acid</td>
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reported that GAS mutants expressing different amounts of hyaluronic acid capsule showed similar virulence in mice [43]. These results suggest the influence of a qualitative, but not quantitative, change in the capsule to be critical in the virulence of GAS. Therefore, we conclude that the hyaluronic acid capsule of GAS is a relatively fundamental virulence factor, although not a limiting factor, in the pathogenesis of GAS infection.

In conclusion, our results indicate that production levels and the gene presence of some virulence traits are determined in an M-type–associated manner. Even though the traits that we have examined have been considered to be important for the development of GAS infectious diseases, our results strongly suggest that the extent of the contribution of each trait to the pathogenesis of GAS infections varies considerably among different M types. Our results also strongly suggest that it is necessary in the investigation of GAS infection to consider the influence of M type differences among GAS.

References


