Protection against Group A Streptococcus by Immunization with J8–Diphtheria Toxoid: Contribution of J8- and Diphtheria Toxoid–Specific Antibodies to Protection

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A conformationally constrained, minimally conserved peptide from the M protein of group A streptococcus (GAS) has been defined. It consists of 12 amino acids from the C-repeat region within a non–M protein helix-forming sequence and is referred to as “J8.” Here, we investigate the immunogenicity of a J8–diphtheria toxoid (DT) conjugate adjuvanted with the human-compatible adjuvants, SBAS2 and alum, and demonstrate that it is capable of inducing opsonic antibodies and can protect outbred mice from virulent challenge. In a range of experiments, protection correlated with the titer of J8-specific antibodies and not with the induction of J8-specific T cells. However, DT-specific antibodies (as well as J8-specific antibodies) were shown to stain the surface of fixed GAS and to be capable of opsonizing live organisms. DT may be an ideal carrier protein for J8 and other GAS peptides for GAS vaccines.

Infection with group A streptococci (GAS) can result in acute and postinfectious pathology, including rheumatic fever (RF) and rheumatic heart disease (RHD). These diseases are associated with poverty and are increasing in incidence, particularly in developing countries and in indigenous populations, such as Australia’s Aborigine population, which has the highest incidence of rheumatic fever worldwide [1]. Immunity to GAS strains is believed to be mediated predominantly by opsonic antibodies to serotypic determinants at the N terminus of the M protein [2]. Vaccine development based on the M protein [3–5] faces a number of significant hurdles. More than 100 serotypes exist, and, although the pathogenesis of RF is not well understood, increasing evidence implicates an autoimmune process that may involve the M protein [6, 7]. Identifying a conserved non-host cross-reactive epitope from the M protein and its role in immunity and defining appropriate human compatible adjuvants for such epitopes is a major strategy in vaccine development.

Previous studies have shown that a collection of peptides from the carboxyl terminal half of the M protein were able to induce heterologous protection in mice [3, 8]. Bessen and Fischetti [3, 9] identified a mixture of 3 synthetic peptides from the C repeat region, that,
when administered to mice, reduced pharyngeal GAS colonization. Similarly, Bronze et al. [8] showed that immunization with either of 2 C repeat region epitopes resulted in increased survival of mice after mucosal challenge with heterologous serotypes of GAS. However, these epitopes occur in a region of the M protein known to be able to induce production of host cross-reactive antibodies and T cells [7, 10]. Furthermore, the mechanism of immunity was not clear from these studies.

We previously identified a conserved region peptide, referred to as “p145,” that was recognized by >90% of Australian Ab-origine serum samples. The presence of antibodies to this peptide increased with age; antibodies were present in ~40% of young children and ~90% of adults [11]. This increase in prevalence with age parallels the acquisition of streptococcal immunity. Human antibodies specific for p145 could directly opsonize multiple GAS strains in vitro and the peptide could induce antibodies in mice that opsonized multiple strains of GAS [11, 12].

We and others have shown that T cell epitopes exist within the carboxyl terminus of the M protein and that some of these are potentially host autoreactive [7, 13, 14]. Our approach, therefore, was to identify the minimal B and T cell epitopes within p145 necessary to develop an immunogen capable of inducing protective antibodies without stimulating potentially harmful T cells.

We identified a minimal, non–host cross-reactive peptide from p145 and displayed this within a non–M protein peptide sequence designed to maintain helical folding and antigenicity [14, 15]. This peptide is referred to as “J8,” and, when admin-
istered with complete Freund’s adjuvant (CFA), it can protect up to 80% of H-2k responder strain mice (B10.BR) from virulent challenge [16]. However, minimal epitopes such as J8 are unlikely to be immunogenic in an outbred population. To render such epitopes immunogenic, they must be conjugated to carrier proteins capable of inducing T cell help. Diphtheria toxoid (DT) has been used as a carrier protein in many systems, and preliminary data from our own laboratory (data not shown) suggested that anti-DT antibodies may, in fact, be capable of opsonizing GAS, making DT an obvious choice for a carrier protein for J8.

The mechanism for J8-mediated protection of mice against GAS challenge has never been defined. In contrast, the N-terminal epitopes of the M protein have been shown to induce high levels of serum antibodies that are highly opsonic to the corresponding GAS strain [5, 17, 18], and these epitopes have also been shown to induce high levels of protection against GAS challenge in mice [16]. In contrast, the conserved region epitope, J8, induces high levels of serum antibodies that have generally lower opsonic activity [14].

The aim of this study was to investigate the immunogenicity of J8-DT in combination with novel and existing adjuvants and to define correlates of protective immunity. Adjuvants of particular interest in this study were CpG [19, 20] and the human compatible adjuvants alum [21, 22] and SBAS2 and SBAS3 [23, 24].

MATERIALS AND METHODS

Peptide synthesis and conjugation to a peptide carrier. Synthetic peptides were produced as described elsewhere [25] and were purified by high-performance liquid chromatography. Peptides were conjugated via a C-terminal cysteine residue to DT (CSL), using 6′-maleimido-caproyl n-hydroxy succinimide (MCS), as described by Coligan et al. [26]. The sequence for the J8 peptide is QAEDKVKQSREAKKQEVAKLQLEDKVQ.

Immunization of mice. Peptides (peptide alone or peptide conjugated to DT) were administered subcutaneously in a volume of 50 μL at the tail base to B10.BR (inbred, H-2k) or Quackenbush (outbred) mice (Animal Resources Centre). Each mouse received a total of 30 μg of immunogen (free peptide or conjugated peptide) emulsified 1:1 in CFA (Difco Laboratories) or adsorbed onto alum (GlaxoSmithKline Biologicales), SBAS2 (SmithKline Beecham Biologicals [SKB]), SBAS3 (SKB), CpG (Genetset Lismore NSW), or a combination of these. The CpG oligonucleotide sequence is 5′-TCC ATG ACG TTC CTG AGG TT-3′. Formulations of the alum, SBAS2, and SBAS3 vac-

cines were prepared by GlaxoSmithKline Biologicals. Selected groups of mice were also given 2 subsequent booster injections at days 21 and 28 after primary immunization. Blood samples were obtained from mice on days 20, 27, and 35 after primary immunization.

Detection of murine antibodies. An ELISA was used to measure J8-specific murine serum IgG antibody titers, and the antibody isotypes were determined as described elsewhere [14, 27]. Titers were defined as the highest dilution to yield gave an optical density (OD) reading of >3 SD above the mean OD of control wells containing serum from healthy mice.

Bactericidal assay. Murine anti-peptide serum samples were assayed for their ability to opsonize GAS in vitro, as described elsewhere [4, 11, 12, 16]. In brief, bacteria were grown overnight at 37°C in 5 mL of Todd-Hewitt broth (THB). GAS was then serially diluted to 10−3 in PBS. For each individual mouse, 50 μL of fresh heat-inactivated serum was mixed with 50 μL of the bacterial dilution and incubated for 20 min at room temperature. After the incubation, 400 μL of nonopsonic heparinized human donor blood was added. All donor blood was prescreened before the assay was done, to ensure that it could support the growth of the GAS strain to at least 32 times the inoculum in a 3-h incubation at 37°C [11].

The mixtures were incubated end-over-end at 37°C for 3 h, and 50 μL from each tube was plated out in duplicate on 2% blood THB agar pour plates. The plates were incubated over-

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night, and the number of colonies on each plate was determined. Opsonic activity of the anti-peptide serum samples (the percentage reduction in mean colony forming units [cfu]) was calculated as $[1 - (\text{mean cfu in the presence of anti-peptide serum})/\text{(mean cfu in the presence of normal mouse serum})] \times 100$.

**GAS challenge procedure.** The M6 GAS strain was passaged through mice to enhance its virulence before challenge. GAS was cultured overnight in THB with 1% Neopeptone (THBN) (Difco Laboratories), washed twice in THBN, and re-suspended in 25% of the original volume. The inoculum dose (cfu/mL) was determined by measuring OD at 600 nm and plating out 10-fold dilutions of bacterial suspensions in 2% horse blood–THB agar. Following overnight incubation at 37°C, colony counts were determined. Immunized and control mice were challenged intraperitoneally with GAS 10 days after the final immunization. According to institutional ethics committee requirements, animals that were observed to be moribund were killed.

**Lymph node cell proliferation assay.** Lymph node cell proliferation assays were performed essentially as described by Pruksakorn et al. [27]. Briefly, the J8 peptide was administered subcutaneously at the tail base, with each mouse receiving 30 µg of peptide emulsified in CFA. Ten days later, lymph node cells were plated in quadruplicate at $4 \times 10^5$ cells/well in Eagle MEM containing $5 \times 10^{-3}$ M β-mercaptoethanol, antigen at various concentrations, and either 2% healthy mouse serum or 5% fetal calf serum. The plates were incubated for 4 days in a humidified 5% CO₂/air environment at 37°C. Each well was then pulsed with 1 µCi of [³H]-thymidine for 16 h. Incorporation of the label was determined by liquid scintillation spectroscopy, using a β-counter (LKB). Stimulation index (SI) was defined as counts per minute in the presence of antigen/counts per minute in the absence of antigen.

**Immunofluorescent assays.** Immunofluorescent assays were used to visualize the interaction between specific antibodies and the streptococcal surface, as described elsewhere [11]. A culture containing GAS in 5 mL of THB/1% neopeptone was incubated overnight and then centrifuged at 1600 g for 10 min, and the pellet was re-suspended in PBS to an OD₅₄₀ of 0.1. Fifty microliters of the bacterial dilution was smeared across polylysine microscope slides (Esco), air-dried at room temperature, and fixed for 10 min with 3% paraformaldehyde (Sigma) in PBS. The slides were washed in PBS (10 min), dried in a humidified chamber at room temperature, placed in sealed containers with silica gel, and stored at −20°C. When required, the slides were thawed at room temperature for 30 min, washed in PBS (10 min), and blocked with 50 µL of 5% skim milk/PBS–Tween 20 for 30 min in a humidified chamber at 37°C. The slides were rinsed in PBS, incubated with nonspecific human IgG (Serotech), diluted 1:200 with 0.5% skim milk in PBS containing 0.05% Tween 20, for 1 h at 37°C in a humidified chamber. The specific primary antibody and fluorescein isothiocyanate (FITC)–conjugated F(ab’)₂ secondary antibody were titrated to determine the optimal concentration for maximum fluorescence intensity. Consequently, 2-mL aliquots of the primary antibody samples, diluted 1:200 with 0.5% skim milk in PBS containing 0.05% Tween 20, were added to each slide and incubated at 4°C overnight in a humidified chamber. Slides were then washed with 2 changes of PBS for 15 min, covered with 1 mL of a 1:100 dilution of FITC-conjugated F(ab’)₂ goat anti–mouse IgG (Silenus), and incubated in the dark at room temperature for 2 h in a humidified chamber. The slides were then washed 3 times in PBS for 30 min and mounted with Vectashield (anti-fade) (Vector Laboratories). Slides were examined using an Axioskop fluorescent microscope at a detection wavelength of 520 nm.

**Statistical analysis.** The geometric mean and the SEM were calculated using standard formulas. The log-rank or Mantel-Haenszel test was used to compare survival curves of mice challenged with GAS. $P < .05$ was considered to be statistically significant.

**RESULTS**

**Immunogenicity of free J8.** The J8 peptide is known to be recognized by T cells from H-2d mice [14]. The immunogenicity of the free J8 peptide, with regard to antibody production, was determined using 5 different adjuvants (CFA, alum, SBAS2, SBAS3, and CpG) and an adjuvant combination of CpG and alum in B10.BR (H-2b) mice. J8-specific IgG end-point titers for the individual mice were determined at day 35 after immunization (figure 1).

All of the J8/adjuvant combinations induced production of IgG antibodies that recognized the J8 peptide; the SBAS2 adjuvant induced the greatest response. None of the adjuvants, when administered alone, induced production of IgG antibodies that recognized the J8 peptide (data not shown). J8 administered with CpG and alum in combination did not lead to significantly higher titers than did J8 with alum alone.

The ability of outbred mice to respond to J8 has not been determined. Immunological responsiveness is largely determined by the ability of T cells to recognize processed fragments of an antigen or, in the case of a small peptide, an entire peptide, in association with an major histocompatibility class II molecule. Lymphocyte proliferation of draining lymph node cells from immunized mice is a standard assay of T cell responsiveness [28]. Inability of J8 to provoke either a T cell or B cell response would limit the suitability of J8 as a vaccine, unless it were conjugated to a carrier protein as a source of T cell help. Quackenbush (outbred) and B10.BR mice were immunized with J8 peptide emulsified in CFA, and lymph node cell
proliferation in response to the peptide was determined for each mouse (figure 2).

Only lymph node cells from 2 of the 20 outbred mice proliferated when stimulated with the J8 peptide (stimulation indices of 4.1 and 4.6). Positive controls (purified protein derivative of *Mycobacterium tuberculosis* and concanavalin A) induced proliferative responses in cells from all Quackenbush mice. In comparison, T cells from 7 of the 8 immunized B10.BR mice responded to J8 peptide. These data confirmed that, for outbred populations, J8 would need to be conjugated to a carrier protein to be rendered immunogenic.

**Conjugated peptide immunization and challenge of inbred and outbred mice using human-compatible adjuvants.** Preliminary data indicated that anti–DT antibodies killed up to 70% of bacteria (in vitro; data not shown). Because of these data, DT was chosen as the carrier protein. The immunogenicity of J8-DT was tested using the human-compatible adjuvants SBAS2 and alum in B10.BR (H-2k) and outbred Quackenbush mouse strains. These 2 adjuvants provided good responses in preliminary studies (figure 1). Two different immunization regimens were used: primary only or primary and 2 boosts. Two different immunogens were used (J8-DT and DT), and 2 doses of immunogen were used (3 and 30 μg). These different combinations were chosen to give a wide range of antibody responses and specificities that would be useful to investigate any correlation between immunogenicity and protection.

All of the J8-DT/adjuvant formulations investigated induced J8-specific serum IgG antibodies in B10.BR mice. J8-DT–immunized mice had opsonic antibodies that were able to kill the M6 GAS reference strain in the in vitro assay (table 1). The mean antibody titers for groups that received 3 μg of the J8-DT immunogen (with and without boosts) were lower than those in the corresponding groups of B10.BR mice that received 30 μg of immunogen (with and without boosts). This was observed with both the SBAS2 and alum adjuvants. Mice that were immunized with 30 μg of DT in either adjuvant developed antibodies that recognized DT (table 1). In a finding consistent with our preliminary data, these antibodies were opsonic in the in vitro assay, in which the M6 GAS reference strain was used, although to a lesser degree than were antibodies from J8-DT–immunized mice. The average opsonic activity of serum from DT-immunized mice ranged from 1.3% to 49.4%, compared with 66.2% to 88.5% for serum from J8-DT–immunized mice (table 1).

Immunized B10.BR mice were challenged with M6 GAS intraperitoneally (table 1 and figure 3). In experiment A, mice immunized with J8-DT and either adjuvant showed significantly greater survival (except for the group that received 3 μg of J8-DT in alum without boosting), compared with the corresponding control groups immunized with PBS (figure 3; *P* < .05). Both of the J8-DT–immunized groups of B10.BR mice in experiment B showed significantly greater survival than did the corresponding PBS/adjuvant control groups (figure 3; *P* < .05). The group of mice immunized with DT (with 2 boosts) had an intermediate level of survival at day 10 that was not significantly different from that in the PBS-immunized group (*P* > .05; figure 3).

Outbred (Quackenbush) mice were immunized with the same formulations and immunization regimen that was used for B10.BR mice. The immunogens again induced production...
of high titer of IgG antibodies that recognized the J8 peptide (table 2). The average opsonic activity for the J8-DT–immunized groups was significantly greater than that for the DT-immunized groups (P < .05); this was also observed among the B10.BR mice (P < .05).

Quackenbush mice were similarly challenged with M6 GAS (table 2 and figure 3). Mice immunized with J8-DT (30 µg) in SBAS2 had a significantly higher rate of survival (P < .05) than did the corresponding PBS-immunized control group at day 10 after challenge (figure 3). None of the groups in which mice received 3 µg of immunogen in SBAS2 had numbers of surviving mice at day 10 after challenge that were significantly different from those in the control groups, although significantly enhanced survival was seen at earlier time points: mice immunized with 3 µg of J8-DT had a significantly greater chance of surviving up to and including day 3 (P < .05) after challenge, compared with the PBS-immunized group.

The J8-DT/SBAS2 combination was associated with significantly greater survival at day 10 after challenge in outbred mice, compared with both the PBS- and the DT-immunized groups (P < .05). Groups of mice that received J8-DT in alum did not have significantly higher rates of survival than did groups that received PBS or DT (P > .05).

Outbred mice immunized with J8-DT in alum also had significantly higher rates of survival after challenge with a non–M typeable GAS strain (88/30) commonly found in the Northern

### Table 1. Serum IgG titers, average opsonization, and survival rates for B10.BR mice immunized against group A streptococcus.

<table>
<thead>
<tr>
<th>Experiment, group</th>
<th>Immunization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>J8-specific IgG GMT&lt;sup&gt;b&lt;/sup&gt; (95% CI)</th>
<th>DT-specific IgG GMT&lt;sup&gt;b&lt;/sup&gt; (95% CI)</th>
<th>Average opsonization, %&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mouse survival, %&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>J8-DT (30 µg) in SBAS2</td>
<td>67,608 (41,687–109,648)</td>
<td>310,418 (189,998–430,838)</td>
<td>86.5</td>
<td>63&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>J8-DT (30 µg) in SBAS2</td>
<td>1,513,561 (870,964–2,630,268)</td>
<td>2,483,350 (1,696,769–3,269,930)</td>
<td>85.8</td>
<td>78&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>J8-DT (3 µg) in SBAS2</td>
<td>7943 (2512–24,547)</td>
<td>102,400 (53,238–151,616)</td>
<td>88.5</td>
<td>50&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>J8-DT (3 µg) in SBAS2</td>
<td>177,828 (102,329–316,228)</td>
<td>356,577 (133,087–680,067)</td>
<td>76.0</td>
<td>50&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>J8-DT (30 µg) in alum</td>
<td>194,987 (3311–114,815)</td>
<td>11,143 (4272–18,013)</td>
<td>83.2</td>
<td>40&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>J8-DT (30 µg) in alum</td>
<td>331,131 (138,038–794,328)</td>
<td>142,631 (546,866–2,305,733)</td>
<td>75.3</td>
<td>70&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>J8-DT (3 µg) in alum</td>
<td>6918 (2692–17,783)</td>
<td>2425 (543–4306)</td>
<td>66.2</td>
<td>33</td>
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<tr>
<td>8</td>
<td>J8-DT (3 µg) in alum</td>
<td>31,623 (6457–154,882)</td>
<td>25,600 (13,309–37,890)</td>
<td>75.7</td>
<td>50&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>DT (30 µg) in SBAS2</td>
<td>115 (93–141)</td>
<td>470,506 (309,946–631,066)</td>
<td>49.4</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>DT (3 µg) in SBAS2</td>
<td>11 (2–74)</td>
<td>356,577 (276,297–436,857)</td>
<td>23.0</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>DT (30 µg) in alum</td>
<td>7 (1–46)</td>
<td>135,117 (85,956–184,278)</td>
<td>17.5</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>DT (3 µg) in alum</td>
<td>49 (11–219)</td>
<td>235,253 (154,973–315,533)</td>
<td>−1.3</td>
<td>11</td>
</tr>
<tr>
<td>13</td>
<td>PBS</td>
<td>1 (1–1)</td>
<td>1 (1–1)</td>
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<td>0</td>
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<td>B</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>J8-DT (30 µg) in SBAS2</td>
<td>190,546 (33,884–1,071,519)</td>
<td>2,852,620 (2,210,380–3,494,860)</td>
<td>65.8</td>
<td>60&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>2</td>
<td>DT (30 µg) in SBAS2</td>
<td>6 (1–35)</td>
<td>3,276,800 (1,098,858–5,454,742)</td>
<td>29.5</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>PBS in SBAS2 + 2 boosts</td>
<td>1 (1–1)</td>
<td>1 (1–1)</td>
<td>0.0</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>J8-DT (30 µg) in alum</td>
<td>660,693 (346,737–1,288,250)</td>
<td>1,426,310 (546,866–2,305,733)</td>
<td>49.2</td>
<td>70&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>DT (30 µg) in alum + 2</td>
<td>2 (1–4)</td>
<td>1,241,675 (848,384–1,634,965)</td>
<td>21.0</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>PBS in + 2 boosts</td>
<td>1 (1–1)</td>
<td>1 (1–1)</td>
<td>0.0</td>
<td>11</td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; DT, diphtheria toxoid; GMT, geometric mean titer.

<sup>a</sup> A similar immunization regimen was used for both adjuvants (SBAS2 and alum). Mice (n = 10) received a primary immunization of either 30 or 3 µg of immunogen (J8-DT), and selected groups then received 2 boosts of J8-DT in PBS on days 21 and 28, at the same concentration used in the primary immunization. Control groups received PBS or DT (30 or 3 µg) in adjuvant.

<sup>b</sup> Determined for serum from the final bleed.

<sup>c</sup> Survival at day 10 after challenge was statistically significantly higher (P < .05), compared with mice that received PBS or PBS in adjuvant.

<sup>d</sup> Survival at day 10 after challenge was statistically significantly higher (P < .05), compared with mice that received 30 µg of DT in SBAS2.

<sup>e</sup> Survival at day 10 after challenge was statistically significantly higher (P < .05), compared with mice that received 30 µg of DT in alum.
Figure 3. Survival (%) among mice after intraperitoneal challenge with the M6 group A streptococcus (GAS) reference strain (not all groups shown; see tables 1 and 2) for B10.BR (experiments A and B) and outbred (experiment A) mice. Outbred mice were also challenged with the 88/30 GAS strain (experiment B). DT, diphtheria toxoid.
Table 2. Serum IgG titers, opsonization, and survival rates for Quackenbush mice immunized against group A streptococci (GAS).

<table>
<thead>
<tr>
<th>GAS, experimental group</th>
<th>Immunization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>J8-specific IgG GMT&lt;sup&gt;b&lt;/sup&gt; (95% CI)</th>
<th>DT-specific IgG GMT&lt;sup&gt;b&lt;/sup&gt; (95% CI)</th>
<th>Average opsonization, %&lt;sup&gt;b&lt;/sup&gt; Mouse survival, %</th>
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<tr>
<td>M6</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>J8-DT (30 µg) in SBAS2</td>
<td>1,148,154 (812,831–1,659,587)</td>
<td>235,253 (114,833–355,673)</td>
<td>80.2</td>
</tr>
<tr>
<td>2</td>
<td>J8-DT (30 µg) in SBAS2 + 2 boosts (30 µg)</td>
<td>1,300,000 (924,436–1,612,538)</td>
<td>2,161,881 (156,487–4,167,275)</td>
<td>79.3</td>
</tr>
<tr>
<td>3</td>
<td>J8-DT (3 µg) in SBAS2</td>
<td>501,187 (223,872–1,148,154)</td>
<td>235,253 (114,833–355,673)</td>
<td>76.9</td>
</tr>
<tr>
<td>4</td>
<td>J8-DT (3 µg) in SBAS2 + 2 boosts (3 µg)</td>
<td>1,884,032 (1,089,296–2,890,451)</td>
<td>485,994 (254,323–717,665)</td>
<td>74.4</td>
</tr>
<tr>
<td>5</td>
<td>J8-DT (30 µg) in alum</td>
<td>501,187 (158,489–1,148,154)</td>
<td>19,401 (13,256–25,546)</td>
<td>78.6</td>
</tr>
<tr>
<td>6</td>
<td>J8-DT (30 µg) in alum + 2 boosts (30 µg)</td>
<td>1,478,630 (1,346,740–1,645,650)</td>
<td>1,882,027 (918,667–2,845,387)</td>
<td>87.3</td>
</tr>
<tr>
<td>7</td>
<td>J8-DT (3 µg) in alum</td>
<td>151,356 (38,019–602,560)</td>
<td>16,889 (122,2–32,556)</td>
<td>83.2</td>
</tr>
<tr>
<td>8</td>
<td>J8-DT (3 µg) in alum + 2 boosts (3 µg)</td>
<td>2,019,952 (1,454,709–2,715,352)</td>
<td>44,572 (17,090–72,054)</td>
<td>74.9</td>
</tr>
<tr>
<td>9</td>
<td>DT (30 µg) in SBAS2</td>
<td>2 (1–5)</td>
<td>540,470 (343,825–737,115)</td>
<td>13.4</td>
</tr>
<tr>
<td>10</td>
<td>DT (3 µg) in SBAS2</td>
<td>1 (1–1)</td>
<td>270,235 (171,912–366,557)</td>
<td>9.0</td>
</tr>
<tr>
<td>11</td>
<td>DT (30 µg) in alum</td>
<td>22 (4–129)</td>
<td>102,400 (52,183–151,561)</td>
<td>9.0</td>
</tr>
<tr>
<td>12</td>
<td>DT (3 µg) in alum</td>
<td>2 (1–4)</td>
<td>204,800 (106,473–303,122)</td>
<td>34.3</td>
</tr>
<tr>
<td>13</td>
<td>PBS</td>
<td>1 (1–1)</td>
<td>1 (1–1)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

NOTE. CI, confidence interval; DT, diphtheria toxoid; GMT, geometric mean titer.

<sup>a</sup> A similar immunization regimens was used for both adjuvants (SBAS2 and alum). Mice (n = 10) received a primary immunization of either 30 or 3 µg of immunogen (J8-DT), and selected groups then received 2 boosts of J8-DT in PBS on days 21 and 28, at the same concentration used in the primary immunization. Control groups received PBS or DT (30 or 3 µg) in adjuvant.

<sup>b</sup> Determined for serum from the final bleed.

<sup>c</sup> Survival at day 10 after challenge was statistically significantly higher (P<.05), compared with mice that received PBS.

<sup>d</sup> Survival at day 10 after challenge was statistically significantly higher (P<.05), compared with mice that received 30 µg of DT in SBAS2.

Territory of Australia than did outbred mice immunized with PBS in alum (P = .02; table 2 and figure 3). This result indicates that the conserved region epitope can induce a protective immune response in outbred mice against different GAS strains.

**Immunofluorescent studies of J8 antisera–binding to GAS.** To explore a possible reason why DT-specific antibodies are opsonic to GAS and partially protective, we used immunofluorescent studies to examine whether DT-specific antibodies could bind the surface of GAS. We demonstrated that serum from the J8/CFA-, J8-DT/SBAS2–, and DT/SBAS2-immunized mice contained antibodies that were able to bind the M6 GAS strain (figure 4). The control group immunized with PBS in adjuvant did not contain antibodies that recognized the bacteria. Furthermore, the DT/SBAS2 antisera also bound to an M1 GAS strain, which indicates that the cross-reactivity observed may not be M type specific. Mice immunized with DT in alum also produced antisera that were capable of binding M6 GAS, which indicates that this reaction was independent of the adjuvant used (data not shown).

**Correlates of immunity.** The enhanced protection observed for B10.BR mice, compared with outbred mice (whose T cells were unresponsive to J8; figure 1), in combination with the low opsonic activity of serum from J8-DT–immunized mice, compared with serum from mice immunized with N-terminal serotypic determinants [16, 18], led us to ask whether J8-specific antibody levels correlated with enhanced survival. Antibody levels (measured by ELISA) for the various groups of immunized mice were plotted against percentage survival for the group after challenge. Both B10.BR and Quackenbush strains were examined. A positive correlation with Quackenbush mice, whose T cells do not respond to J8 (figure 2), would support the argument that effector T cells were not involved in protection.

Linear regression analysis of the B10.BR data yielded R² =
Figure 4. Photomicrographs (representative of the slide) of immunofluorescent (fluorescein isothiocyanate)–stained group A streptococci (GAS) viewed under the same conditions, using a microscope with a 100× objective. Bacteria appeared as pairs or chains of cocci ∼0.5 μm in diameter. A, M6 GAS exposed to antisera from mice immunized with J8/complete Freund’s adjuvant. B, M6 GAS exposed to diphteria toxoid (DT)/SBAS2 antisera. C, M1 GAS exposed to DT/SBAS2 antisera. D, M6 GAS exposed to J8-DT/SBAS2 antisera. E, M6 GAS exposed to PBS/SBAS2 antisera. F, Bright-field view of E.

0.5651 and P<.02 (figure 5A), which indicates that there is an overall positive relationship between mean J8-specific serum IgG titer and percentage survival in J8-DT–immunized mice. An overall positive correlation was also observed between mean J8-specific serum IgG titer and percentage survival for the Quackenbush challenge experiment; linear regression analysis yielded $R^2 = 0.4499$ and $P<.05$ (figure 5B).

DISCUSSION

We have previously investigated the potential of combining the conserved region peptide into novel self-adjuvanting lipid constructs for use as an intranasal vaccine [29]. Immunization with these lipid-core constructs induced opsonic antigen-specific serum IgG, and mice were significantly protected after challenge with an M1 GAS strain [29, 30]. However, although these lipid-core constructs have enormous potential as intranasal vaccines, this technology is still in early development with regard to any human application. In addition, these experiments were conducted using B10.BR mice, which are known to have immunological responses to the peptide J8 in the presence of adjuvant, without carrier help.

The aims of this study were to determine the immunogenicity of a conserved M protein epitope, J8, with various adjuvants, to identify correlates of protection, and to investigate the potential of the conjugated peptide (J8-DT) as a GAS vaccine candidate using human compatible adjuvants. We have demonstrated that J8 and J8-DT induced significant antibody responses when delivered subcutaneously with human compatible adjuvants and that immunized mice demonstrated significantly enhanced survival against different strains of GAS. The degree of protection correlated with the ELISA antibody titer.

Of the adjuvants tried with the free J8 peptide, SBAS2 was superior. The SBAS2-adjuvanted formulation induced higher IgG antibody responses than did CFA-, alum-, SBAS3-, or CpG-adjuvanted vaccines. The CpG bacterial DNA motif has been shown to play a role in murine B cell activation, stimulation of cytokines, and induction of specific antibodies [31]. As an adjuvant, CpG has been shown to induce strong peptide-specific cytotoxic T lymphocyte activity in draining lymph nodes [32]. Furthermore, CpG appears to switch the isotype pattern to a Th1 profile, with IgG2a becoming the dominant isotype [32, 33]. CpG has also been successfully used as a mucosal adjuvant with purified hepatitis B virus surface antigen, when administered alone and when given with cholera toxin [34]. Although CpG may be a suitable mucosal adjuvant, we found in this study that J8 administered subcutaneously with CpG or CpG and alum induced antigen-specific titers that were significantly less than those induced by J8/CFA.

The SBAS2 adjuvant consists of an oil-in-water emulsion...
combined with monophosphoryl lipid A and QS-21 (saponin derivative) [24]. This adjuvant was designed to induce very strong antibody responses, strong effector cell immune responses (Th1, primarily), and major histocompatibility class I–restricted cytotoxic T lymphocyte responses.

SBAS2 has been reported to be more potent than alum but also more reactogenic [24]. It is, however, still under investigation for widespread use in humans for the prevention or treatment of infectious and noninfectious diseases, such as malaria, hepatitis B, human papillomavirus infection, and cancer [24]. SBAS2 has successfully been used in conjunction with the \textit{Plasmodium vivax} apical membrane antigen 1 in rhesus monkeys [35] and has also been combined with the malaria vaccine candidate RTS,S in a human phase 1 safety and immunogenicity trial [36].

Ling et al. [23] reported that mice immunized with a recombinant malaria protein (\textit{Plasmodium falciparum} merozoite-specific protein 1) in SBAS2 developed a strong IgG response in which the major isotype was IgG1, followed by IgG2a and IgG2b. Overall, the SBAS2 adjuvant induced a stronger total IgG and isotype response than did CFA.

In comparison, the human-approved alum (aluminum hydroxide) adjuvants induce a Th2-type response, with high levels of IgG1 and IgE antibodies [37–39]. The main use of alum is to induce a humoral response with protein antigens; however, this adjuvant is not particularly well suited to use with unconjugated peptide antigens [40].

Problems with peptide conjugation to carriers can result when amino acids essential to the antigenic determinant are altered or obscured during conjugation [41]. In the present study, we demonstrated the feasibility of conjugating the J8 conserved region epitope to DT. The conjugation of the J8 peptide via a C-terminal cysteine residue using MCS did not destroy the essential conformational structure of the peptide; antibodies from mice immunized with J8-DT in a variety of adjuvants were able to recognize free J8 peptide.

DT alone was shown to induce serum that was capable of killing the M6 GAS in vitro. DT also induced intermediate protection against GAS challenge in B10.BR mice when it was administered with both SBAS2 and alum. A search of the GAS genome database [42] revealed that several proteins contain short regions that are partially homologous with DT, in particular, members of the ABC transporter lipoprotein family. Thirty-six ABC transporters were identified from the GAS genome, and many of these are associated with conserved membrane transport systems [42]. ABC transporters are a large family of proteins responsible for translocation of a variety of compounds across biological membranes [43].

DT also contains several $\alpha$-helical regions that may contain conformational epitopes that could induce antibodies that cross-react with the strong $\alpha$-helical structure of the M protein or other surface proteins of GAS. The third helical region of DT (residues 346–372) resembles the transmembrane helical regions of intrinsic membrane lipoproteins [44, 45]. DT also contains 2 other $\alpha$-helical domains, at residues 207–225 and 242–264 [44]. These homologous or conformational regions of the DT protein may induce cross-reactive antibodies that are capable of opsonization of GAS in the in vitro assay and intermediate protection against GAS challenge in vivo. DT may be an ideal carrier protein for a GAS vaccine that is based on J8 or other GAS peptides.

An overall positive correlation was observed in the 4 challenge experiments between average J8-specific serum IgG titer and the percentage of survival. In addition, T cells from the majority of J8-immunized Quackenbush mice did not proliferate in the presence of J8 peptide, which suggests that the J8-DT–induced protection from GAS challenge is mediated by J8-specific antibodies and not by T cells. This mechanism of immunity may be facilitated by particular IgG isotypes, and it is believed that, in mice, the IgG2a isotype is important for the opsonization of bacteria [46]. However, in the present study, there was no independent correlation of protection with IgG2a titers (data not shown).

Different adjuvants induce different immunological responses, and it is, therefore, important to develop a human vaccine formulation that will induce a high-titer opsonic an-

Figure 5. Scattergram and linear regression analysis of mouse survival rates (%) and J8-specific serum IgG titers after challenge with group A streptococci for B10.BR (A) and Quackenbush (B) mice immunized with J8–diphtheria toxoid.
tibody response. These preclinical studies have shown the feasibility of conjugating J8 to DT and that this conjugated peptide can be successfully combined with adjuvants compatible for human use. Our data suggest that the conjugated peptide from the conserved region of the M protein could form the basis of a broad-based antistreptococcal vaccine.

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References


