Infection with group A streptococcus (GAS) may result in a number of human diseases, including potentially life-threatening postinfectious sequelae. In the present study, J14, a conformationally constrained conserved minimal peptide from the M protein, was incorporated into a lipopeptide construct to which a universal T cell epitope and a self-adjuvanting lipid moiety, PamCys, were also attached. We demonstrate that this lipopeptide construct, when administered intranasally (i.n.) without additional adjuvants, protects outbred mice from lethal respiratory GAS challenge. In addition, the lipopeptide was capable of inducing J14-specific mucosal immunoglobulin A, which coincided with reduced throat colonization after respiratory GAS challenge. These preclinical experiments show that this lipopeptide could form the basis of an antidisease and transmission-blocking i.n. GAS vaccine.

Infection with group A streptococcus (GAS) can result in a number of clinical manifestations, ranging from relatively benign, self-limiting pharyngitis to invasive diseases, such as necrotizing fasciitis. Although these diseases create a huge economic burden for both developing and developed nations, of greater concern are the postinfectious manifestations, rheumatic fever (RF) and rheumatic heart disease (RHD). RF/RHD is an autoimmune disease in which T cells and antibodies induced by GAS virulence factors (such as the M protein) are thought to play a role in pathogenesis by cross-reacting with human tissues [1].

The development of a vaccine to prevent GAS infection and its associated diseases has primarily focused on the M protein, an abundant cell-surface virulence factor. Two areas of the M protein have been the target of vaccine development: the hypervariable N-terminal region [2] and the conserved C-terminal region [3]. We have previously defined a conformationally constrained conserved minimal peptide, J14, that does not contain a potentially deleterious T cell epitope from the M protein [4]. This epitope consists of 14 aa from the C-
repeat region of the M protein that is contained within a non-streptococcal helix-promoting sequence that has been derived from the DNA-binding protein GCN4 [4]. Such a molecule mimics the natural conformation in which the conserved 14 aa residues occur. More recently, we have demonstrated that this peptide can induce a protective immune response in mice against both intraperitoneal and intranasal (inl) challenge with heterologous GAS strains [5, 6].

A primary route of GAS infection in humans is colonization of the mucosal epithelium of the pharynx. Colonization followed by tissue invasion can lead to local suppurrative complications or systemic infection [1]. On mucosal surfaces such as the mucosal epithelium, IgA is one of the host's primary defense mechanisms for preventing bacterial infection and for inhibiting the binding of bacteria to epithelial cells [7]. Human salivary IgA specific for p145, a conserved-region peptide of the M protein, is able to opsonize heterologous strains of GAS [8]. In early human clinical trials of the M protein [9], patients immunized inl with the M protein had lower frequencies of both throat colonization and clinical illness than did patients vaccinated systemically, indicating the importance of a local immune response in protection against GAS infection. Taken together, these data strongly suggest that the preferred route of immunization for the prevention of GAS infection is inl; however, there are, at present, no adjuvants available that are suitable for the administration of peptides in humans.

It has been previously demonstrated that passively acquired IgA directed against the M protein is able to significantly inhibit GAS infection in mice when administered mucosally [10]. Furthermore, mice immunized inl with synthetic peptides from the conserved region of the M protein in the presence of the mucosal adjuvant cholera toxin B subunit (CTB) showed a significant decrease in the incidence of pharyngeal GAS colonization [3]. Although CTB has been widely used as an inl adjuvant in numerous animal models, immune responses and tolerance of this adjuvant have varied among different species. In addition, CTB and other enterotoxin-based adjuvants have been recently shown to accumulate in the olfactory nerves and bulbs of mice after inl administration, with associated evidence of inflammation of the olfactory region of the brain [11]. Moreover, enterotoxin-based adjuvants have severe diarrheagenic properties when ingested in small amounts [12]. Therefore, the safety of enterotoxin-based adjuvants for human use has been questioned.

Previous studies investigating inl adjuvants have demonstrated that lipids of known adjuvanticity can be covalently linked to peptides to make immunogenic lipopeptides that can generate both antibody and cellular immune responses [13]. These fully synthetic lipopeptide constructs are potentially safe vaccines; the self-adjuvanting lipopeptides have not induced adverse effects in phase 1 human clinical trials [14].

A variety of lipid moieties have been studied for the purpose of vaccine design. Pam$_2$Cys, a synthetic version of the lipid moiety from the 2-kDa macrophage-activating lipopeptide 2 derived from Mycoplasma fermentans, has been found to have very potent adjuvanting activity [13] and to target the Toll-like receptors (TLRs) on dendritic cells [15]. We have used this lipid moiety in previous experiments analyzing the construction of self-adjuvanting lipopeptide-based vaccines [15]. A generic structure is used in which a colinear T cell epitope and either a target B cell epitope or a cytotoxic T lymphocyte epitope are separated by an intervening lysine residue to which Pam$_2$Cys is attached via its ε-amino group [15]. We found that such branched peptides were not only more soluble, compared with N-terminal–attached lipids, but were also more immunogenic.

As part of the search for a highly immunogenic and totally synthetic inl GAS vaccine formulation that would be suitable for human use and that would also overcome the unresponsiveness of the conserved-region peptide in a genetically diverse population, we constructed a lipopeptide containing J14, a conformationally constrained conserved minimal peptide that does not contain the potentially deleterious T cell epitope from the M protein; a universal T cell epitope that has been used in vitro and in vivo across different species [15, 16]; and the Pam$_2$Cys lipid. These components were assembled together in the same branched geometry (figure 1) that we have used previously [15, 17].

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**Figure 1.** Schematic representation of the peptide constructs used in the present study. LHRH, luteinizing hormone–releasing hormone.
MATERIALS AND METHODS

Peptide synthesis. All peptide constructs (figure 1) were synthesized using standard FMOC chemistry. Lipidation and purification of lipopeptides was done as described elsewhere [15]. For the 2 lipopeptides, P25-P2C-J14 and P25-P2C–luteinizing hormone–releasing hormone (LHRH), the T cell epitope P25 (sequence, GKLIPNASLIENCTKAEL), which had been derived from the fusion protein of the morbillivirus canine distemper virus [16], was synthesized continguously and N-terminally to either the peptide J14 (sequence, KQAEDKVASREAKQVEKALEQLEDVKV) or the control peptide LHRH (sequence, HW5GYLRPG). In both cases, Pam₃Cys was attached to the ε-amino group of the intervening lysine residue placed between the 2 epitopes.

Inl immunization of mice. Outbred Quackenbush mice (Animal Resources Centre, Western Australia) to be immunized inl were mildly anesthetized with a mixture of xylazine and ketamine (1:1:10 mixture of xylazine:ketamine:H₂O; Provet). Each mouse (n = 14–45/group) was administered 60 µg of the P25-P2C-J14 formulation or its derivatives in a total volume of 30 µL (15 µL/nare). The mice received 2 booster immunizations on days 21 and 42 after primary immunization in the same fashion and dose as the primary immunization. All animal experimental work was done in accordance with the guidelines of the Queensland Institute of Medical Research Animal Ethics Committee.

Serum, saliva, and fecal collection. Blood was collected from mice via the tail artery and allowed to clot for at least 30 min at 37°C. Serum was collected after centrifugation for 10 min at 1000 × g, heat inactivated for 10 min at 56°C, and stored at −20°C. Serum was collected from all of the mice 1 day before the booster immunizations and 15 days after the final booster immunization.

Mice were administered 50 µL of a 0.1% solution of pilocarpine (Ioquin), to induce salivation. Saliva was then collected and mixed with 2 µL of 50 mmol/L phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Sigma). Particulate matter was separated by centrifugation for 10 min at 13,000 × g, and samples were stored at −70°C.

Six to 10 freshly voided fecal pellets were collected from individual mice, frozen at −70°C, and lyophilized. The dry weight of fecal solids was determined before they were resuspended by vortexing in 5% nonfat dry milk, 50 mmol/L EDTA, 0.1 mg/mL soybean trypsin inhibitor (Sigma), and 2 mmol/L PMSF (20 µL/mg of dry weight). Solid matter was separated by centrifugation for 10 min at 15,000 × g. The supernatants were stored at −70°C until analyzed.

Determination of antibody titers. An ELISA was used to measure J14-specific murine serum IgG and mucosal IgA titers essentially as described elsewhere [4]. A titer was defined as the highest dilution that gave an optical density reading >3 SDs above the mean optical density of control wells, which contained normal mouse serum. Statistical significance (P < .05) was determined using nonparametric analysis of variance with Dunn’s posttest analysis.

Opsonophagocytic assay. Murine antiserum was assayed for its ability to opsonize GAS in vitro, as described elsewhere [18, 19]. For each mouse, 50 µL of fresh heat-inactivated serum was mixed with 50 µL of a bacterial dilution (1 × 10⁻⁵) and incubated for 20 min at room temperature. After incubation, 400 µL of nonopsonic heparinized human donor blood was added. The mixtures were incubated end over end at 37°C for 3 h, and 50 µL from each tube was plated in duplicate on 2% blood Todd-Hewitt agar pour plates. The plates were incubated overnight, and the number of colony-forming units was determined.

Procedure for inl GAS challenge. Immunized and control mice were challenged inl with a predetermined dose of the GAS strain M1. To determine GAS colonization, throat swabs were obtained from mice on days 1, 2, 3, 6, 9, and 15 after challenge. The throat swabs were streaked out on Todd-Hewitt agar plates containing 2% horse blood and incubated overnight at 37°C. A swab was classified as positive when a plate had 1 or more GAS colony-forming units. The statistical significance (P < .05) of differences between groups after GAS challenge was determined by the Mantel-Haenszel log-rank test for survival-curve analysis and by the χ² test for throat-swab status.

RESULTS AND DISCUSSION

We have previously demonstrated that the GAS M protein conserved-region peptide alone does not induce a T cell response in a majority of outbred Quackenbush mice and that the immune response is restricted to mice of the H-2b background [5]. We asked whether immunization with constructs containing Pam₃Cys would also lead to a genetically restricted response. Apart from PBS, the nonlipidated peptide J14, and P25-J14, we also used a lipidated peptide construct, P25-P2C-LHRH, as a control. Although this construct has the same branched geometry as P25-P2C-J14, the J14 B cell epitope is replaced with the peptide hormone LHRH. We observed that inl immunization (primary plus 2 booster immunizations; 60 µg each) of outbred Quackenbush mice with the lipopeptide construct P25-P2C-J14 induced antibodies in all of the mice tested (figure 2A). As was expected, the antibody titer increased after each booster immunization (data not shown). On day 60 after primary immunization, the mice immunized with either P2C-J14 or P25-P2C-J14 had significantly higher (P < .05) J14-specific serum IgG titers than did the mice in the control groups, including the mice administered P25-P2C-LHRH. Furthermore, the mice immunized with P25-P2C-J14 had higher responses than did the mice immunized with P2C-J14.

To investigate the efficacy of the antiserum and to determine

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whether vaccination had the potential to prevent GAS infection, the opsonic activity of the serum on day 60 after primary immunization was determined using an in vitro assay that measures colony-forming units of GAS. Inl immunization with P25-P2C-J14 induced serum that elicited significantly greater ($P < .05$) levels of in vitro opsonization of the GAS strain M1 than did administration of PBS or P25-P2C-LHRH, whereas opsonization by serum from mice immunized with P2C-J14 was not significantly different from that of the PBS or P25-P2C-LHRH control groups (figure 2B). However, there was marked variability in the responses of the mice immunized with P2C-J14, which may have resulted from the fact that the mice were outbred.

One of the main reasons for the use of an inl vaccine is its ability to induce a local immune response at the primary point of GAS infection. Therefore, we were interested in determining whether the J14 lipopeptide constructs were capable of inducing peptide-specific mucosal IgA. We found that the mice immunized inl with P2C-J14 or P25-P2C-J14 had significantly great-
Figure 3. Percentage of dead, throat-swab–positive, and throat-swab–negative mice in each group on days 1, 2, 3, 6, 9, and 15 after challenge with the group A streptococcus strain M1. A significantly higher \((P<.05)\) no. of mice immunized with P25-P2C-J14 were throat-swab negative on day 15 after challenge, compared with the no. of mice administered J14 alone, P25-J14, P25-P2C–luteinizing hormone–releasing hormone (LHRH), or PBS. The mice immunized with P25-P2C-J14 also had a significantly higher \((P<.05)\) survival rate, compared with the mice administered J14 alone, P25-J14, P25-P2C-LHRH, or PBS.

We further determined J14-specific salivary IgA titers than did the mice in the control groups (figure 2C). We further determined J14-specific mucosal IgA titers in fecal samples collected from the immunized mice. The mice immunized with either P2C-J14 or P25-P2C-J14 had significantly greater \((P<.05)\) J14-specific fecal IgA titers than did the control mice immunized with J14 alone, P25-J14, P25-P2C-LHRH, or PBS (figure 2D).

The immunized mice were challenged inl with the GAS strain M1 on day 63 after primary immunization (figure 3). To monitor GAS colonization of the throat during the 15-day postchallenge observation period, throat swabs were obtained on days 1, 2, 3, 6, 9, and 15 after challenge. At the end of the observation period (day 15 after challenge), there was a significantly greater \((P<.05)\) number of surviving mice in the group immunized with P25-P2C-J14, compared with the number of surviving mice in the control groups (figure 3). It is of considerable interest that P2C-J14 administered inl induced low but significant levels of IgG and that the mice that received P2C-J14 demonstrated enhanced survival after challenge. The data raise the possibility that deep-tissue infection with GAS (which is frequently lethal) may be preventable by a vaccine that induces relatively low titers of J14-specific serum IgG.

The mice immunized with P25-P2C-J14 demonstrated a significant decrease in colonization, as measured by the percentage of mice that either (1) continued to be throat-swab positive for GAS or (2) failed to survive for the full 15 days after challenge (figure 3). Interestingly, the P25-P2C-J14 and P2C-J14 constructs that decreased colonization also induced significantly higher titers of salivary and fecal IgA, compared with those induced by J14 alone, P25-J14, P25-P2C-LHRH, and PBS (figure 2C and 2D).

The predominant antibody at mucosal surfaces is the secretary form of IgA, which has been shown to play a major role in the prevention and control of mucosal bacterial infections. To induce a strong IgA response, it is particularly important to target mucosa-associated lymphoid tissue, such as the nasal-associated lymphoid tissue in rodents [20], because these structures sample antigens directly from the epithelial surface and are a source of primed B cells for the various secretory effector sites. Previous work has demonstrated that the major antigen-presenting cells involved in the uptake of lipopeptides are not macrophages but dendritic cells, which are responsible for the priming of the immune system at both the systemic and mucosal levels [12]. We have more recently shown that the mechanism of Pam_Cys uptake by dendritic cells is mediated by TLR-2 [15], indicating a possible mechanism of action for the lipid adjuvant.

The ability of the P2C-J14 construct to induce antibodies without the inclusion of the T cell epitope P25 was of interest. The addition of a hydrophobic lipid tail to peptides has previously been shown to be moderately immunogenic. Peptides derived from the Plasmodium falciparum circumsporozoite protein, when covalently linked to a lauroyl lipid anchor group, induced serum IgG after immunization in outbred mice [21]. The control lipopeptide P25-P2C-LHRH has the same branched geometry and incorporates the same T cell epitope and lipid moiety as the lipopeptide P25-P2C-J14 but elicited no J14-specific antibody responses or opsonic activity and failed to
protect mice from GAS challenge. This finding indicates that the J14-specific neutralizing antibody activity contributed to the prevention/therapeutic activity of the vaccine tested and that the T cell epitope P25 only provided help to generate J14-specific antibody and did not directly contribute to the therapeutic effects of the vaccine. Interestingly, we also observed a significant correlation between the average level of opsonization within a group of immunized mice and the percentage of mice that survived the GAS challenge ($r = 0.6870; P = .0066$), a finding that also highlights the possible contribution of neutralizing antibody activity to protection.

The lipopeptide construct P25-P2C-J14, which contains a conformationally constrained conserved minimal peptide, a universal T cell epitope, and a self-adjuvanting lipid moiety, is totally synthetic and easily characterized. When administered in I.D., this lipopeptide was able to overcome genetic restriction and induced J14-specific antibody responses in outbred mice at both the systemic and mucosal levels. By combining our conserved-region peptide with a universal T cell epitope and the self-adjuvanting lipid Pam$_2$Cys, we were able to induce significant levels of J14-specific serum IgG that not only significantly protected mice from respiratory GAS challenge but that was also capable of inducing significant levels of mucosal IgA in the outbred mice that coincided with a significant reduction in GAS throat colonization, indicating that this lipopeptide construct would not only protect against systemic infection but may also act a transmission-blocking vaccine.

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