Toward the Development of an Antidisease, Transmission-Blocking Intranasal Vaccine for Group A Streptococcus

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Infection with group A streptococcus (GAS) may result in a number of clinical conditions, including the potentially life-threatening postinfectious sequelae of rheumatic fever and rheumatic heart disease. As part of the search for a vaccine to prevent GAS infection, a conformationally constrained and minimally conserved peptide, J14, from the M protein of GAS has been defined. In the present study, J14 was formulated with bacterial outer membrane proteins (proteosomes) and then intranasally administered to outbred mice without additional adjuvant. Such immunization led to high titers of J14-specific serum immunoglobulin (Ig) G and mucosal IgA. After upper respiratory tract GAS challenge, immunized mice demonstrated increased survival and reduced GAS colonization of the throat.

Infection with group A streptococcus (GAS) may result in a number of human diseases, including the potentially life-threatening postinfectious sequelae of rheumatic fever (RF) and rheumatic heart disease (RHD). RF/RHD is an autoimmune disease in which it is thought that T cells and antibodies induced by GAS virulence factors (such as the M protein) play a role in pathogenesis by cross-reacting with human tissues [1]. In humans, the primary route of GAS infection is colonization of the mucosal epithelium of the pharynx. Colonization followed by tissue invasion can lead to local suppurrative complications or systemic infections [1]. IgA is one of the host’s first lines of defense against the prevention of bacterial infection and inhibits the attachment of bacteria to the mucosal epithelium. Human salivary IgA specific for p145, a conserved-region peptide of the M protein, has previously been shown to opsonize GAS in vitro [2]. Moreover, in early human clinical trials that used the M protein, subjects who were immunized intranasally (inl) with complete M protein had both reduced throat colonization and reduced clinical illness, compared with those who were vaccinated systemically, indicating the importance of a local immune response in protection against GAS infection [3]. In addition, it has been demonstrated previously that passively acquired IgA specific for the M protein is able to significantly inhibit GAS infection in mice when administered mucosally [4]. Taken together, these data strongly suggest that the preferred route of immunization for the prevention of GAS infection is inl administration.

As part of the search for a safe vaccine to prevent GAS infection and the postinfectious autoimmune complication RF/RHD, a conformationally constrained and minimally conserved peptide, J14, from the M protein that does not contain a potentially deleterious T cell autoepitope has been defined [5]. The J14 peptide consists of 14 aa from the C-repeat region of the M protein.
within a nonstreptococcal helix-promoting sequence. Such a molecule mimics the natural conformation in which the conserved 14 aa occur. To overcome the immunological nonresponsiveness of the J14 peptide in a genetically diverse population, we investigated the feasibility of a proteosome adjuvant system, which has demonstrated a good safety profile as well as immunogenicity and protection in several phase 1 and 2 clinical trials of a proteosome-based nasal influenza vaccine [6]. Proteosomes consist of outer membrane proteins (OMPs) extracted from phenol-killed group B type 2 Neisseria meningitidis [6]. We hypothesized that the addition of the minimal B cell epitope from the M protein with the proteosome vesicle would result in a vaccine formulation that can overcome the peptide’s immunological unresponsiveness in a genetically diverse population and also induce a local mucosal immune response that would reduce GAS colonization after upper respiratory tract challenge.

MATERIALS AND METHODS
cJ14/proteosome construct. To promote noncovalent complexing of J14 to proteosomes, a hydrophobic anchor consisting of a fatty acid lauroyl chain was added to the epsilon-amino moiety of the lysine near the J14 carboxyl terminus (cJ14). In addition, a cysteine (to promote peptide dimerization) and a tripeptide spacer were added near the lauryl moiety.

Inl immunization of mice. Quackenbush (outbred) mice (Animal Resources Centre, Western Australia) to be immunized inl were mildly anesthetized by use of a mixture of xylazine (1:1:10 mixture of xylazine:ketamine:H2O). Mice were administered either the cJ14/proteosome construct or cJ14 alone in a volume of 30 μL (15 μL/naris); control mice were administered a proteosome-only formulation. The mice received 2 booster immunizations 21 days apart in the same fashion as the primary immunization. All animal experimental work was conducted in accordance with the recommendations of the Queenland Institute of Medical Research Animal Ethics Committee.

Serum, salivary, and fecal sample collection. Blood was collected from the mice via the tail artery and was allowed to clot for at least 30 min at 37°C. Serum was obtained by centrifugation at 1000 g for 10 min; it was then heat inactivated (10 min at 56°C) and stored at −20°C. Blood was collected from all of the mice 1 day before each booster immunization and 15 days after the final booster immunization.

To monitor mucosal immune responses, groups of mice were salivated. Briefly, the mice were administered 50 μL of a 0.1% solution of pilocarpine (The Ioquin Company), to induce salivation. Saliva was then collected and mixed with 2 μL of 50 mmol/L phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Sigma). Particular matter was separated by centrifugation at 13,000 g for 10 min, and the samples were stored at −70°C.

Six to ten freshly voided fecal pellets were also collected from each mouse; the pellets were frozen at −70°C and then lyophilized. The dry weight of the fecal solids was determined, and the solids were resuspended (1 mg/mL) in 5% nonfat dry milk, 50 mmol/L EDTA, 0.1 mg/mL soyabean trypsin inhibitor (Sigma), and 2 mmol/L PMSF protease inhibitor (20 μL/mg of dry weight), by vortexing. Solid matter was separated by centrifugation at 15,000 g for 10 min. The supernatants were stored at −70°C until they were analyzed.

Determination of antibody titers. An ELISA was used to measure J14-specific serum, fecal, and salivary IgG and IgA titers, essentially as described elsewhere [5, 7]. A titer was defined as the highest dilution that gave an optical density reading of >3 SDs above the mean optical density of control wells, which contained normal mouse serum.

Opsonization assay. Murine peptide antiserum was assayed for its ability to opsonize GAS in vitro, as described elsewhere [8–12]. 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 the incubation, 400 μL of nonsonicized heparinized human donor blood was added. The mixtures were incubated end-over-end for 3 h at 37°C and 50 μL from each tube was plated in duplicate on 2% blood Todd–Hewitt broth (THB) agar pour plates (Oxoid). The plates were incubated overnight, and the number of colonies on each plate was counted.

Procedure for inl GAS challenge. Before inl GAS challenge, the bacterial isolate M1 was cultured overnight in THB with 1% neopeptone and 200 mg/mL streptomycin, was spun at 1500 g for 10 min, and was washed twice in THB with 1% neopeptone before being resuspended in 25% of the original volume. The inoculum dose (colony-forming units per milliliter) was determined by reading the optical density at 600 nm and plating out 10-fold dilutions of bacterial suspensions on 2% horse blood THB agar pour plates. After overnight incubation of the plates at 37°C, the number of colonies on each plate was counted. The immunized and control mice were challenged inl with the predetermined dose of GAS in a volume of 30 μL (15 μL/naris). To determine GAS colonization, the throats of the mice were swabbed on days 1, 2, 3, 6, 9, and 15 after challenge. Swabs were streaked out on THB agar pour plates containing 2% horse blood, which were incubated overnight at 37°C. A swab was classified as positive when a plate had ≥1 cfu. The GAS reference strain, M1, was obtained from the Menzies School of Health Research (Darwin, Australia). This isolate has been sequence typed, passaged though mice to enhance virulence, and selected for streptomycin resistance (200 μg/mL).

Lymph node cell proliferation assay. The lymph node cell
proliferation assay was performed essentially as described by Pruksakorn et al. [7]. Briefly, J14 or cJ14 was administered subcutaneously (sc) at the tail base, with each mouse receiving 30 μg of peptide with complete Freund’s adjuvant (CFA) or proteosomes. Ten days later, lymph node cells were plated in quadruplicate at 4 × 10⁵ cells/well in Eagle MEM containing 5 × 10⁻³ mol/L β-mercaptoethanol, antigen at various concentrations, and 2% normal mouse serum. The plates were incubated for 4 days at 37°C in a humidified 5% CO₂/air environment. Each well was then pulsed with 1 μCi of [³H]-thymidine for 16 h. Incorporation of the label was determined by liquid scintillation spectroscopy with a beta counter (LKB). A stimulation index (SI) was defined as the counts per minute in the presence of antigen divided by the counts per minute in the absence of antigen.

**Statistical analysis.** All statistical calculations were performed by use of GraphPad Prism (version 4.01; GraphPad Software). For the ELISA data, nonparametric analysis of variance with Dunn’s post test was used to determine the statistical significances of differences between groups. For the inl GAS challenge data, survival curve analysis with the Mantel-Haenszel log rank test and the χ² test was used to determine the statistical significances of differences between groups. P < .05 was considered to be statistically significant.

**RESULTS**

We initially examined the immunogenicity of the cJ14/proteosome construct administered sc in outbred mice. We have previously demonstrated that J14 alone does not induce an immune response in the majority of outbred mice and is restricted to mice of the major histocompatibility complex (MHC) H-2k background [13]. We report similar data here after measuring T cell activation and antibody production in mice immunized with the different J14 peptides (J14 and cJ14) administered with CFA or proteosomes (figure 1A and 1B). The positive controls for the lymph node cell proliferation assays (purified protein derivative of *Mycobacterium tuberculosis* [PPD] for immunization with CFA and concanavalin A [conA] for immunization with proteosomes) induced proliferative responses (SI >3.0) from T cells from all immunized mice. Lymph node cells from only 1 of 3 mice immunized with J14 with CFA proliferated when stimulated with J14. Likewise, lymph node cells from only 1 of 5 outbred mice immunized with cJ14 with CFA proliferated when stimulated with J14. Similarly, sc immunization with J14 or cJ14 formulated with CFA induced an antibody response in only 3 of 5 outbred mice (figure 1B).

In contrast, sc immunization with the cJ14/proteosome construct (figure 1B) or inl immunization (primary plus 2 booster immunizations) with the cJ14/proteosome construct (without additional adjuvant) (figure 2A) induced antibodies in all mice tested. On day 60 after primary immunization, J14-specific serum IgG titers were significantly greater in the mice immunized with the cJ14/proteosome construct, compared with those in the control mice immunized with proteosome alone. These data suggest that the universal antibody responses of the proteosome-immunized mice most likely resulted from

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**Figure 1.** A, Stimulation index (SI) from the lymph node cell proliferation assay for Quackenbush (outbred) mice after immunization with J14 or cJ14 [see Materials and Methods for definition] administered subcutaneously at the tail base, with each mouse receiving 30 μg of peptide with complete Freund’s adjuvant (CFA) or proteosome. Proliferation of the cells induced by J14 (1 and 3 μg) and, as controls, by purified protein derivative of *Mycobacterium tuberculosis* (PPD) and concanavalin A (conA) was determined. The mean counts per minute in the absence of antigen for lymph node cells from the control mice immunized with proteosome alone was 151.2.

**Figure 2.** B, Individual J14-specific serum IgG titers in outbred mice after subcutaneous immunization with various formulations of J14 and with controls.
Figure 2. A, Mean J14-specific serum IgG titer of Quackenbush (outbred) mice on day 60 after primary intranasal (inl) immunization. The mice immunized with the cJ14/proteosome construct (n = 15; see Materials and Methods for definition) had significantly higher serum IgG titers than did the control mice immunized with proteosome alone (n = 10). The mice immunized with cJ14 alone (n = 10) developed moderate serum IgG titers. The mice received booster immunizations on days 21 and 42 after primary immunization. Statistical significance (considered to be P < .05) was determined by nonparametric analysis of variance with Dunn’s post test.

B, Mean opsonization of M1 group A streptococcus (GAS) by serum collected on day 60 after primary immunization. Mice were immunized with the cJ14/proteosome construct, proteosome alone, or cJ14 alone. The mean colony-forming unit count from the opsonization assay for the mice immunized with the cJ14/proteosome construct was 1420 cfu (range, 944–1712 cfu); in contrast, the mean colony-forming unit counts were 2428 cfu (range, 1824–3040 cfu) for the mice immunized with proteosome alone and 1964 cfu (range, 1792–2224 cfu) for the mice immunized with cJ14 alone. Normal mouse serum supported the growth of GAS by at least 32 times the inoculum during the 3-h incubation at 37°C.

C, Mean J14-specific salivary IgA titers on day 59 after primary immunization for mice immunized with the cJ14/proteosome construct, proteosome alone, or cJ14 alone. The mice immunized with the cJ14/proteosome construct had significantly higher titers than did the other immunized groups (P < .05).

D, Mean J14-specific fecal IgA titers on day 46 after primary immunization. Titers were not determined for the control mice immunized with proteosome alone. E, Percentage of dead, nasal-swab positive, and nasal-swab negative mice in each group on days 1, 2, 3, 6, 9, and 15 after inl challenge with M1 GAS. There was a significant difference in the number of mice that were nasal-swab positive vs. nasal-swab negative between days 1 and 15 after challenge for the mice immunized with the cJ14/proteosome construct. Statistical significance was determined by the Mantel-Haenszel log rank test and the χ² test. Asterisks indicate statistically significant differences.
T cells specific for the OMPs in the proteosome, which provided help to the B cells specific for J14 [14].

To determine whether immunization had the potential to prevent GAS infection, the opsonic activity of serum on day 60 after primary immunization was determined by an in vitro assay that measures GAS colony-forming units, as described elsewhere [12]. In immunization with the cJ14/proteosome construct induced antibodies that elicited significant levels of in vitro opsonization of M1 GAS, whereas opsonization by serum from mice immunized with cJ14 alone was not significantly different from that of the control mice immunized with proteosome alone (figure 2B).

We next examined mucosal immunity, protection from challenge, and protection from colonization. Significant levels of J14-specific IgA were detected in both the salivary and fecal samples from all of the mice immunized with the cJ14/proteosome construct but not from the mice in any other group (figure 2C and 2D). Interestingly, none of the mice in any group developed significant levels of J14-specific salivary IgG (data not shown). To evaluate survival, immunized mice were challenged intranasally with M1 GAS (figure 2E). At the end of the observation period (day 15 after challenge), there was significantly greater survival in the mice immunized with cJ14 alone or with the cJ14/proteosome construct than in the control mice immunized with proteosome alone (figure 2E).

It is of considerable interest that cJ14 administered intranasally without proteosomes induced low but significant levels of IgG and that the mice immunized with cJ14 alone demonstrated enhanced survival after challenge. The data suggest that deep-tissue infection with GAS (which is frequently lethal) may be prevented by a vaccine that induces relatively low levels of J14-specific IgG in serum.

To measure the effects of vaccination on GAS colonization, the throats of the mice were swabbed regularly throughout the 15-day observation period (figure 2E). The data showed that the mice immunized with the cJ14/proteosome construct demonstrated a significant decrease in colonization, as measured by the percentage of mice that either (1) continued to test throat-swab positive for GAS or (2) failed to survive for the full 15 days after challenge.

All of the control mice immunized with proteosome alone or cJ14 alone were either dead or throat-swab positive on day 15. In contrast, 53% of the mice immunized with the cJ14/proteosome construct were throat-swab negative on day 15, and this percentage was trending upward when the protocol was completed. The cJ14/proteosome construct was the only vaccine that induced significantly higher levels of salivary and fecal IgA, compared with those in the control mice immunized with proteosome alone (figure 2C and 2D).

DISCUSSION

In the present study, we demonstrated that immunization with the cJ14/proteosome construct resulted in decreased GAS colonization in mice after challenge and induced both salivary and fecal J14-specific IgA at levels higher than those in control mice immunized with proteosome alone. This is consistent with previous data demonstrating that inl immunization with heat-killed whole GAS organism can protect mice against both heterologous and homologous GAS infection [15]. In addition, it has also been demonstrated that passively acquired IgA specific for the M protein coadministered with GAS can protect mice from inl challenge [4].

We have previously demonstrated that affinity-purified salivary IgA specific for the peptide p145 can opsonize an M5 GAS strain in vitro [2] and that this immunoglobulin can fix complement in the presence of GAS. Moreover, p145-specific salivary IgA is prevalent in an Aboriginal population living in a region in which GAS is endemic and has been shown to increase with age, which is consistent with the acquisition of natural immunity in these regions [2]. The J14 peptide used in the present study contains a minimal B-cell epitope from the larger p145 peptide [5] that is based on the conserved region of the M protein.

These data, combined with the observation from the present study that J14-specific salivary IgA reduces M1 GAS colonization of the throat, highlight the importance of IgA in the control of GAS colonization. Therefore, the induction of salivary IgA responses after inl immunization is an important aspect of GAS vaccine development, because IgA may play several critical roles in the control of GAS colonization of the throat—such as opsonization of GAS and prevention of the attachment of the bacteria to host cells—and may reduce overall transmission between hosts.

This is the first study to investigate the immunogenicity and protective potential of the conserved-region J14 peptide combined with proteosomes. The addition of the lipid tail to the peptide was critical, because it allowed the peptide to be incorporated into the proteosome vesicle. This peptide/proteosome construct, when administered to outbred mice, was able to induce both a systemic and a mucosal immune response, therefore overcoming the immunological nonresponsiveness of this restricted peptide. Although this has overcome a significant problem, of more interest is the induction of both the systemic and local mucosal immune responses after inl immunization. We believe that J14-specific serum IgG provides systemic protection and that mucosal IgA reduces GAS transmission, as was observed in this study. The mice immunized with the cJ14/proteosome construct had significantly reduced mortality, compared with the control mice immunized with proteosome alone; this may be attributable to the systemic
serum IgG controlling the infection, or it may have been a consequence of reduced GAS colonization of the throat and subsequent systemic infection.

The hydrophobic nature of the proteosomes is believed to aid in antigen uptake by antigen-presenting cells and macrophages. Although OMPs have long been known to be B cell mitogen and polyclonal activators in mice and humans, they have recently been shown to stimulate Toll-like receptor (TLR) 2, with resultant up-regulation of B7.2 MHC class I and II ligands on antigen-presenting cells. Consequently, proteosome adjuvants are now considered to be a prime example of the family of adjuvants that stimulate immunogenicity by activating the TLR system and can, thus, fulfill the roles of both peptide carrier and adjuvant [6].

By combining the J14 peptide with proteosomes, we have successfully demonstrated the potential for an nI vaccine to be immunogenic in an outbred population and to prevent GAS colonization and infection. The proteosomes, which are compatible for human use, can act as both a peptide carrier and an adjuvant. The present preclinical experiments indicate that the cJ14/proteosome construct could form the basis for an antidisease, transmission-blocking nI GAS vaccine.

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**References**