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Design of Three-Component Vaccines against Group A Streptococcal Infections: Importance of Spatial Arrangement of Vaccine Components

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Immunological assessment of group A streptococcal (GAS) branched lipopeptides demonstrated the impact of spatial arrangement of vaccine components on both the quality and quantity of their immune responses. Each lipopeptide was composed of three components: a GAS B-cell epitope (J14), a universal $CD4^+$ T-cell helper epitope (P25), and an immunostimulant lipid moiety that differs only in its spatial arrangement. The best systemic immune responses were demonstrated by a lipopeptide featuring the lipid moiety at the lipopeptide C-terminus. However, this candidate did not achieve protection against bacterial challenge. The best protection (100%) was shown by a lipopeptide featuring a C-terminal J14, conjugated through a lysine residue to P25 at the N-terminus, and a lipid moiety on the lysine side chain. The former candidate features α-helical conformation required to produce protective J14-specific antibodies. Our results highlight the importance of epitope orientation and lipid position in the design of three-component synthetic vaccines.

Introduction

Success of the development of a group A streptococcal (GAS[a]) vaccine is expected to save 517 000 deaths per annum according to a recent independent review commissioned by the World Health Organization and would offer an ideal means to prevent rheumatic heart disease and other GAS-associated diseases.[1] Currently available methods of prevention are either inadequate or ineffective as shown by the morbidity and mortality associated with this pathogen worldwide. Although research toward an effective vaccine has been carried out over 70 years, a commercial vaccine is still not available. Application of traditional vaccine approaches (using killed or live attenuated GAS) was limited because of the cross-reactivity of antibodies and T-cells elicited against GAS cell surface M-protein, which is thought to be associated with the development of serious postinfection diseases.[2,3]

Apart from the human tissue cross-reactivity, M-protein can be considered as a good candidate for subunit vaccine design against GAS because of its importance for GAS colonization and invasion (major virulent factor) as well as the high opsonic properties of anti-M-protein antibodies.[4] Structurally, M-protein has an α-helical coiled-coil conformation with a conserved C-terminus.[5] Therefore, peptides derived from the M-protein C-terminus offer a means to develop broadly protective vaccines without inducing cross-reactive antibodies.

Investigation of the C-terminus of the M-protein led to identification of a short peptide (20mer) known as p145 which elicited opsonic antibodies but may still have potential to induce cross-reactive T-cells.[6] Mapping the p145 sequence for a minimal B-cell epitope devoid of any potential cross-reactivity revealed two short peptides (12mer and 14mer).[7] These peptides were found to be linear and did not retain the native α-helical conformation which prevents their binding to antibodies elicited against the GAS M-protein.[7]

The chimeric peptide (J14) (KQAEDKVK**ASREAKKQVEKALE**QLEDKVK) was designed to incorporate the minimal B-cell epitope (14 amino acids shown in bold) enclosed within α-helix-promoting sequences to mimic the native M-protein conformation.[8,9] Antibodies raised against J14 were reported to opsonize 33 out of 37 GAS isolates from an endemic area (79% coverage) including strains that did not contain amino acid sequence identical to J14.[10]

The inclusion of a minimal epitope in a vaccine may result in a restricted immune response among a genetically diverse population. The use of a promiscuous $CD4^+$ T-helper peptide epitope covalently linked to B-cell epitopes can ameliorate the immune response in a genetically diverse population.[11,12] Similarly, attachment of various lipids to peptide-based vaccines has been extensively studied as a successful strategy to improve immunogenicity of short peptides.[11,13] These lipids target toll-like receptors (TLR) expressed by immune cells resulting in robust immune responses.[14]

Covalent linkage of the three components (TLR ligands and B- and T-cell epitopes) results in rapid and long lasting immune responses that exceed the result of their physical mixtures. This led to an emerging synthetic vaccine approach "three-component vaccines" against many infectious diseases and cancers for which traditional approaches failed.[12,15,16]

In a previous publication,[17] we proposed self-adjuvanting lipopeptide vaccine candidates composed of three components: a GAS B-cell epitope (J14), a universal $CD4^+$ T-cell helper epitope (P25) derived from canine distemper virus,[18] and an immune-stimulant lipid moiety targeting toll-like

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[a] Abbreviations: CD, circular dichroism; CFA, complete Freund's adjuvant; CTB, cholera toxin subunit B; GAS, group A streptococcal; IgG, immunoglobulin G; Laa, lipoamino acid; PBS, phosphate buffered saline; *p*MBHA, *p*-methylbenzhydrylamine.

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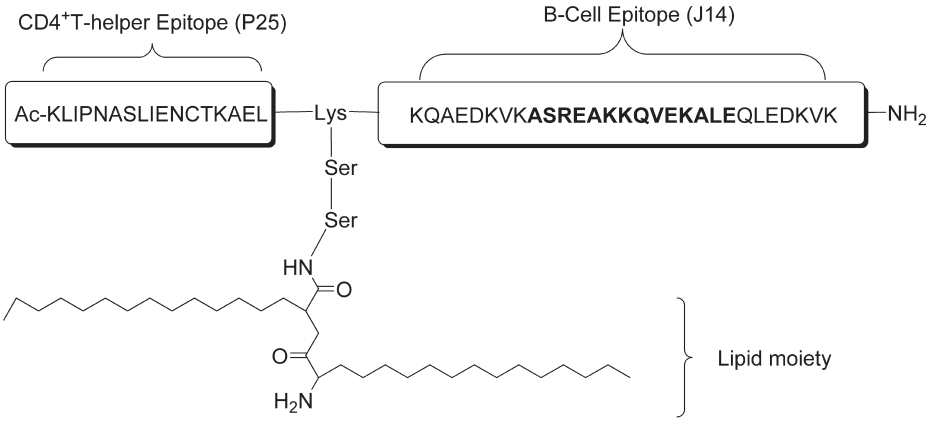
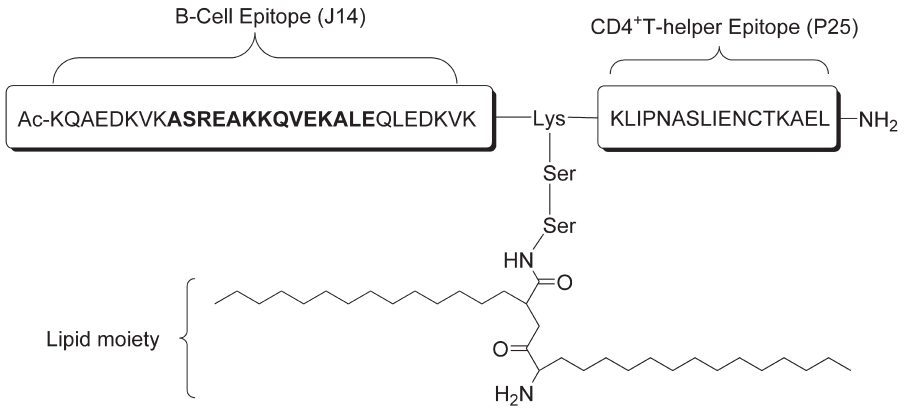
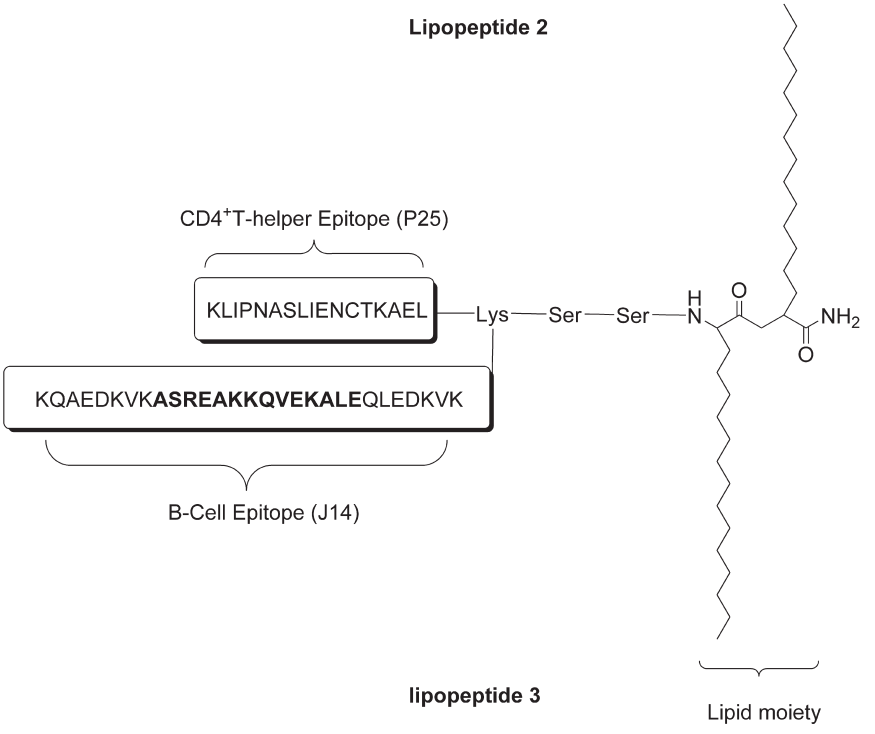


Lipopeptide 1



Lipopeptide 2



lipopeptide 3

Figure 1. Structure of the three-component lipopeptide vaccine candidates **1**−**3**. Lipopeptides incorporate a universal helper T-cell epitope (P25), a GAS B-cell epitope (J14), and a built-in adjuvant based synthetic lipoamino acids (2-amino-D,L-hexadecanoic acid).

receptor 2.[19] We observed that the spatial arrangement of the three components (orientation of the peptide epitopes and lipid moiety) affects the quantity of immune responses (levels of antibody titers). In the current study, we introduce a new aspect of the design of "three-component" vaccines. The study reports correlations between spatial arrangements of the

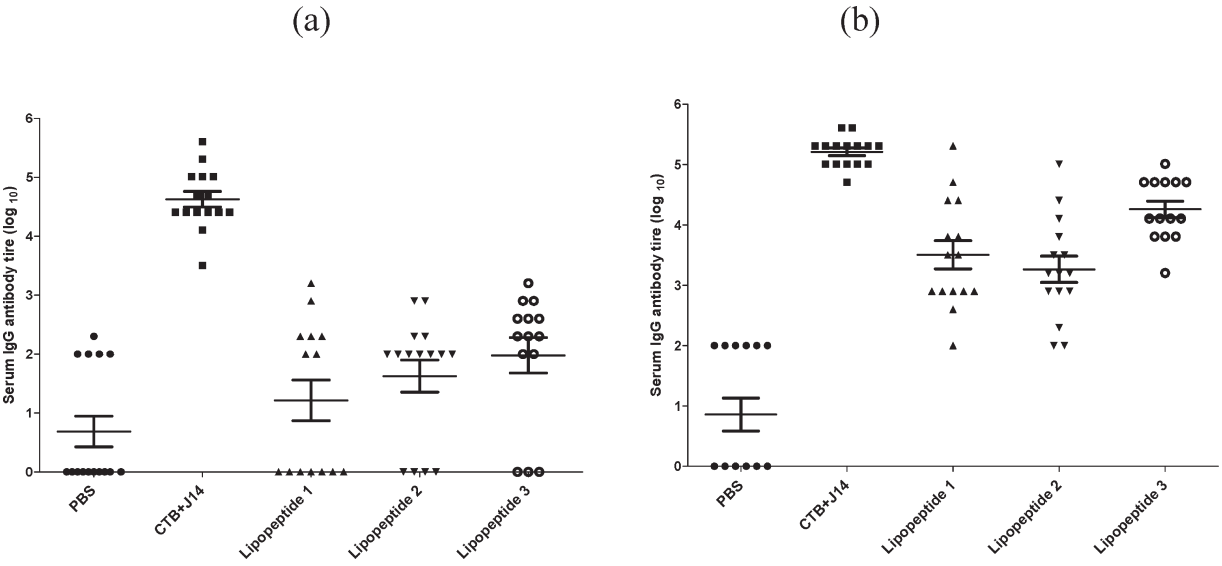


Figure 2. J14-specific serum IgG antibody titers ($\log_{10}$) at day 40 (left) and day 50 (right) elicited in response to intranasal immunization of B10.BR (H-2^k) mice with lipopeptides, as determined by ELISA. Antibody titers are shown for individual mice to the J14 GAS peptide epitope. Mean antigen-specific serum IgG antibody titers are represented as a bar. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison test.

vaccine components and both the vaccine secondary structure (conformation) and the quality of immune responses (efficacy of immune responses). Three lipopeptides (**1**−**3**, Figure 1) were investigated that have the same components (J14, P25, and lipid) but differ only in their spatial arrangement. Immunological evaluation was performed following intranasal administration of these lipopeptides to B10.BR (H-2^k) mice to assess their ability to elicit systemic immune responses without an additional adjuvant. Immunized mice were challenged with the virulent GAS M1 strain to assess the ability of our lipopeptides to protect mice against intranasal GAS challenge. The avidity of antibodies elicited by the three lipopeptides toward the native GAS sequence (p145) as well as the conformation of lipopeptides was assessed to explain the subtle differences in their immune responses.

Results and Discussion

A library of 17 lipopeptide GAS vaccine candidates described in ref 17 were investigated in a small scale experiment (5 mice per group) to select promising candidates that demonstrated the best local and systemic J14-specific antibodies following intranasal administration (unpublished results). Three lipopeptides (**1**−**3**, Figure 1) were selected that were the most immunogenic (J14-spefici antibody titer) in the small scale experiment and were further investigated in a larger experiment (15 mice per group) followed by challenging vaccinated mice intranasally with virulent GAS M1 strain. Immunological evaluation was performed in B10.BR (H-2^k) mice following intranasal priming by lipopeptides **1**−**3**, without an additional adjuvant. The positive control group was administered J14 emulsified with cholera toxin subunit B (CTB), and the negative control group was administered sterile-filtered phosphate-buffered saline (PBS). Mice received two boosts of each immunogen at days 21 and 41 postprimary immunizations. Sera were collected prior to each boost, and 9 days after the final boost, to assess the levels of J14-specific IgG using ELISA.[8]

The selected lipopeptides represent different constructs that would provide information about the effect of varying epitope orientation (J14 and P25) and lipid position on immune

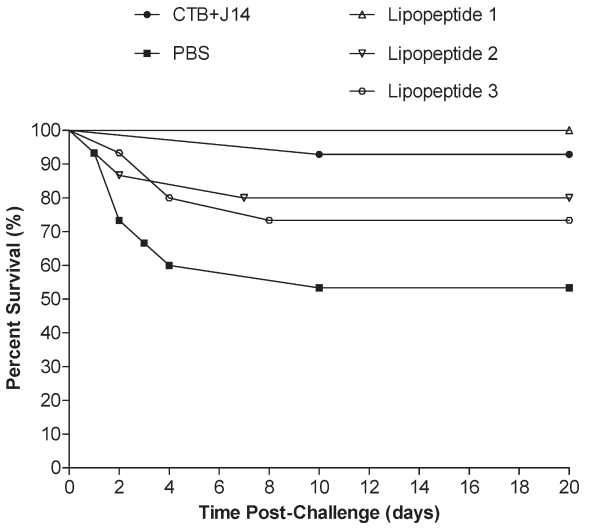


Figure 3. Survival curve of B10.BR mice ($n = 15$) following intranasal immunization with lipopeptides, PBS, and CTB + J14 and challenge with group A streptococcal strain M1.

responses. Lipopeptide **3** and CTB + J14 mixture were able to elicit early significant J14-specific serum immunoglobulin G (IgG) titers on day 40 (Figure 2a, **3** and CTB + J14 vs PBS, $p < 0.05$ and $p < 0.001$, respectively). Other lipopeptides (**1** and **2**) elicited statistically nonsignificant levels of IgG response on day 40 after primary immunization (Figure 2a, **1**, **2** vs PBS, $p > 0.05$). However, systemic IgG antibody titers increased quickly after the third inoculation of each lipopeptide, with the highest antibody titers observed in the case of lipopeptide **3** (Figure 2b, day 50, **3** vs PBS, $p < 0.001$, and vs CTB + J14, $p < 0.05$). The level of systemic IgG antibodies elicited in response to immunization with all lipopeptides was significantly greater than the titers in the negative control groups ($p < 0.001$), suggesting that a primary and two boosts of the lipopeptides are required to induce significant antibody response. These results demonstrated the superior immunogenicity of lipopeptide **3** over other lipopeptides, which

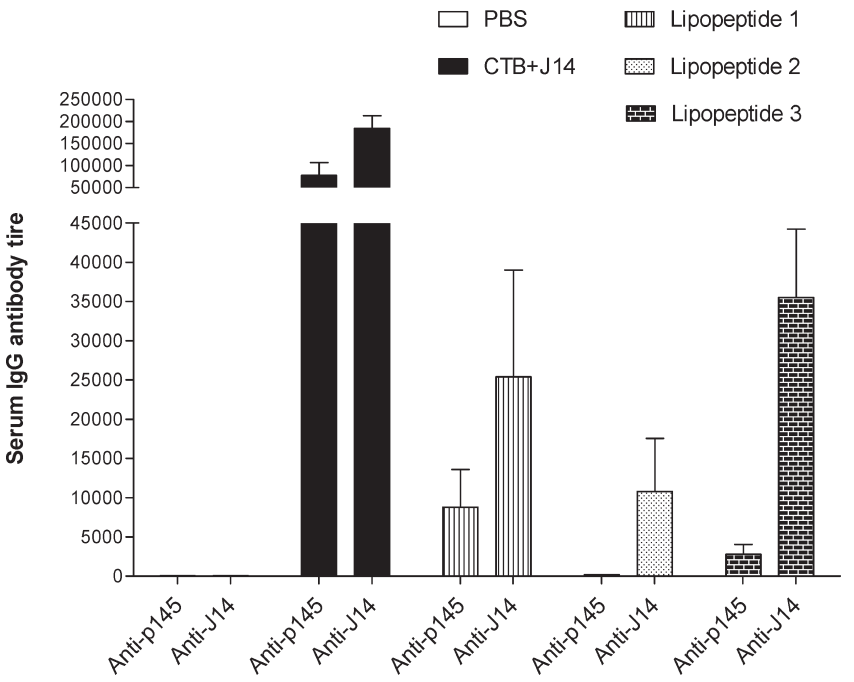


Figure 4. J14 vs p145-specific serum IgG antibody titers at the final bleed (day 50) elicited in response to intranasal immunization of B10.BR (H-2^k) mice with lipopeptides, as determined by ELISA. Mean antigen-specific serum IgG antibody titers are represented as a bar. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison test.

matches previous observations following subcutaneous immunization.[11,17] Overall, the point of lipid moiety attachment had the greatest influence on systemic J14-specific IgG antibody titers following intranasal administration.

Immunized mice were challenged via intranasal route with a virulent GAS M1 on day 56 after primary immunization where morbidity was observed during the first 3 weeks postchallenge (Figure 3). Mice immunized with lipopeptide **1** achieved the highest protection (100%) in 3 weeks after the challenge, which was significantly greater than the negative control group and lipopeptide **3** (**1** vs PBS and **3**, $p < 0.01$ and $p < 0.05$, respectively). Neither lipopeptide **2** nor **3**, which demonstrated higher systemic J14-specific antibody titers, was able to achieve any statistically significant protection different from the negative control group in the 3 weeks after the challenge (**2** and **3** vs PBS, $p > 0.05$).

The second observation concerning the ability of these lipopeptides to protect mice against GAS challenge does not match with the order or magnitude of specific J14 IgG antibody titers elicited by these lipopeptides (Figure 2). This led us to investigate the quality of antibody responses produced against administered lipopeptides.

In order to assess the quality of IgG antibodies produced against the incorporated J14, we assessed the avidity of systemic IgG antibodies toward the native p145 GAS sequence. This was also investigated as a possible reason to explain the differences in the ability of tested lipopeptides to protect mice against GAS infection. The peptide (p145) is the minimal native sequence (20mer) derived from the C-terminus of the M-protein of GAS M1 strain. A comparative avidity of systemic IgG antibodies elicited by tested lipopeptides at day 50 toward both J14 and p145 was investigated in Figure 4.

Systemic IgG antibodies elicited by lipopeptide **2** were incapable to bind p145 (Figure 4, **2** vs PBS, $p > 0.05$). Meanwhile, systemic IgG antibodies elicited by both lipopeptides **1** and the CTB + J14 mixture displayed the highest avidity toward p145 (Figure 4, **1** and the CTB + J14 mixture vs PBS, $p < 0.001$) followed by lipopeptide **3** (Figure 4, **3** vs PBS, $p < 0.01$). The order of J14 titers was as follows: CTB + J14 > **3** > **1** > **2**. The order of p145 titers was as follows: CTB+J14 > **1** > **3** > **2**. The order of avidity toward p145 matches the exceptional ability of lipopeptide **1** to protect mice against GAS challenge. To sum up, the ability of antibodies raised by different J14-containing lipopeptides to bind native GAS M-protein derived sequence (p145) was investigated as a means to determine the quality rather than the quantity of antibodies produced against incorporated J14. Different levels of avidity toward p145 demonstrated by tested lipopeptides could be a reason for their ability to protect mice against GAS challenge. These results clearly indicated the effect of epitope and lipid orientation on both quality and quantity of immune response of our three-component vaccine strategy.

The deferential ability of antibodies to bind the native GAS peptide (p145) led us to investigate the conformation of tested lipopeptides. Circular dichroism (CD) is a rapid method used to determine the secondary structure of proteins and to study their folding and binding.[20] It depends on the fact that different conformations of molecules can affect their absorption of left-handed and right-handed circularly polarized light, resulting in characteristic CD spectra. For example, α-helical proteins have negative bands at 222 and 208 nm. CD spectra of the tested lipopeptides solutions in PBS indicated large differences in conformation among these lipopeptides (Figure 5). The only lipopeptide that shows a typical α-helical pattern was lipopeptide **1** (Figure 5a). Lipopeptide **2**, which differs from lipopeptide **1** only in the orientation of their epitopes (J14 and P25), was found to demonstrate highly random coil spectrum, indicating loss of α-helical confirmation. These results indicate clearly the effect of spatial arrangement of synthetic vaccine components (epitope and lipid orientation) on the conformation of peptide-based vaccines, as lipopeptides **1**−**3** contain the same three components and differ only in their

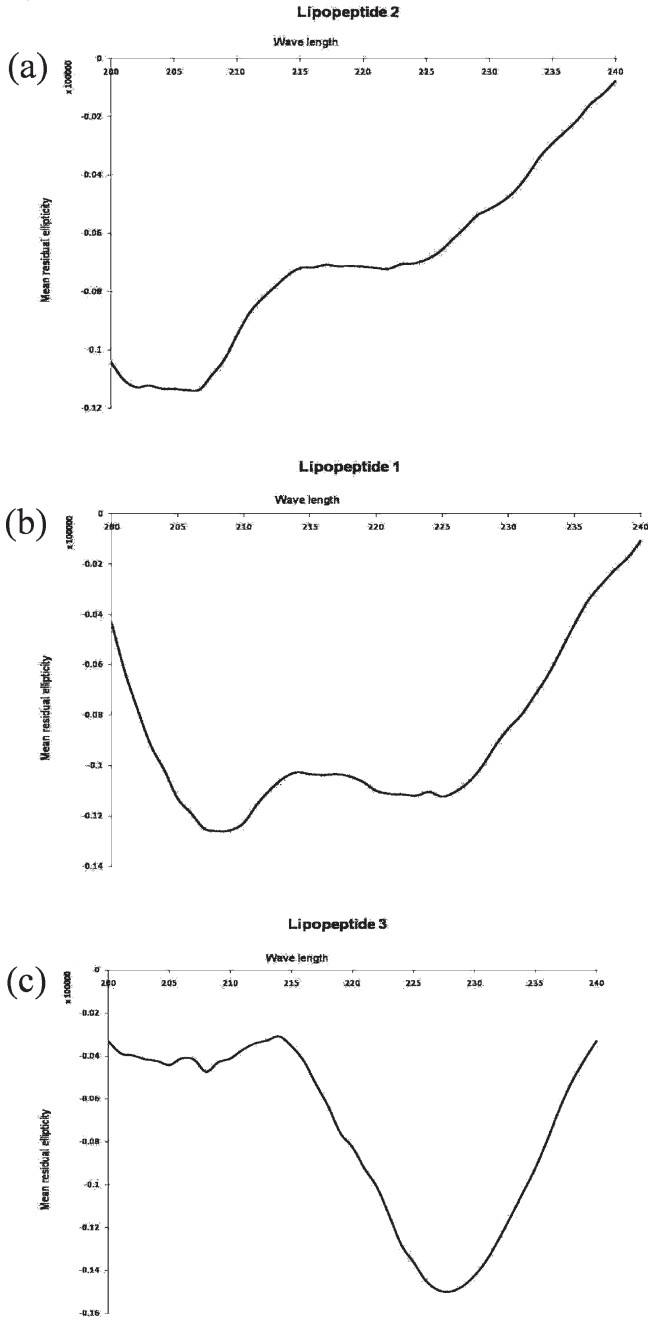


Figure 5. CD spectra of solutions of lipopeptides in PBS. CD spectra were measured in the far UV region (240−200 nm), reported as mean residual ellipticity [θ] in deg cm^2 dmol^{-1}.

orientation. Further investigations are needed to refine structural factors that lead to this big difference in lipopeptide conformation. Nevertheless, the α-helical conformation demonstrated by lipopeptide **1** may explain its ability to elicit the highest titers of conformational antibodies capable of binding the native GAS peptide (p145)

Conclusion

Recent trends in synthetic peptide-based vaccine research focus on the design of multicomponent vaccines by incorporation of minimal bacterial epitopes, T-cell helper epitopes, and lipids. It is classical in this field to observe that incorporation of multiple copies of these epitopes can enhance the quantity of immune response. Except for few reports,[12,17] the effect of varying the spatial arrangement of vaccine components on the level of antibody responses has not been extensively studied. To our knowledge, the current study is the first to demonstrate the importance of the spatial arrangement of vaccine components for the quality of immune responses. By investigation of the structure−activity relationships of lipopeptides following intranasal immunization, it was demonstrated that the orientation of the vaccine peptide-epitopes and the position of lipid attachment may have a great effect on the immune response in terms of level of antibody titers in combination with the avidity toward native bacterial sequence which could account at least in part for protection against infection by our three-component vaccine. Our current study provides two promising lipopeptide GAS vaccine candidates: one that produced strong systemic responses but lacks α-helicity and protection against GAS and the other lipopeptide that demonstrated good protection against GAS but was not as immunogenic. This led to an interesting observation that the spatial arrangement of epitopes and lipids also affects local secondary structure propensity, which was the most unanticipated possibility. Our ongoing research is focused to further refine structural features required to achieve both α-helicity and strong mucosal immune responses shown by previously mentioned lipopeptides and provide a vaccine candidate that combines the two advantages.

Experimental Section

Peptide Synthesis. Lipopeptides **1**−**3** were synthesized, purified, and characterized as previously described.[17] Briefly, each lipopeptide vaccine was synthesized on *p*MBHA resin (0.4 mmol of NH_2/g, 0.5 mmol scale) using manual stepwise solid-phase peptide synthesis, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and *N*,*N*-diisopropylethylamine in situ neutralization, and Boc-chemistry.[21] After the synthesis of each lipopeptide, the peptidyl-resins were cleaved with anhydrous HF. HF was removed under reduced pressure. The peptides were precipitated in ice-cold diethyl ether, filtered, dissolved in 40% aqueous acetonitrile (MeCN) containing 0.1% trifluoroacetic acid (TFA), and lyophilized. The lyophilized products (150 mg) were then purified by preparative RP-HPLC on either a C4 column using a gradient of 10% solvent B (90% MeCN/0.1% TFA/H_2O) to 100% solvent B over 60 min. The fractions were analyzed by ESI-MS, SDS−PAGE, and analytical RP-HPLC and where appropriate combined to give pure product (>95%).

Immunological Assessment of Vaccine Candidates. All protocols were approved by the Queensland Institute of Medical Research Animal Ethics Committee and were carried out according to Australian National Health and Medical Research guidelines.

Intranasal Immunization. Female B10.BR (H-2^k) mice (4−6 weeks old, Animal Resource Centre, Perth, Western Australia, Australia) were used for immunization. Mice (n = 15/group) were anesthetized with a solution of xylazine/ketamine in water (1:1:10, Provet) prior to immunization. Mice received a primary intranasal dose of 60 μg of immunogens (lipopeptides **1**−**3**) dissolved in a total volume of 30 μL (15 μL/nostril) of PBS. Two further boosts (similar to the primary dose) were administered to mice at days 21 and 42. Similarly, the negative control group was administered 30 μL of PBS and the positive control group received three doses of 30 μg of J14-DT mixed with 10 μg of CTB in a total volume of 30 μL of PBS.

Collection of Sera. Blood was collected from the tail artery of each mouse 1 day prior to each dose and 9 days after the last booster immunization. The blood was left to clot at 37 °C for 1 h and then centrifuged for 10 min at 3000 rpm to remove clots. Sera were then stored at −20 °C.

Detection of Systemic IgG by ELISA. Determination of serum IgG antibodies against the J14 or p145 epitopes was performed using a previously described ELISA.[6] Briefly, serial 2-fold

dilutions of samples were produced in 0.5% skim milk/PBS−Tween 20 buffer, starting at a concentration of 1:100 for sera and 1:1 for saliva and feces. Optical density was read at 450 nm in a microplate reader following the addition of peroxidase-conjugated goat anti-mouse IgG (sera). The antibody titer was defined as the lowest dilution with an optical density more than 3 standard deviations greater than the mean absorbance of control wells containing normal mouse sera.

Intranasal GAS Challenge Experiment. Mice were challenged intranasally with a predetermined dose of virulent GAS M1 strain 56 days after primary immunization. Throat swabs were obtained from mice on days 1, 2, 3, 6, 9, and 15 after challenge to determine GAS colonization. Throat swabs were streaked out on Todd−Hewitt agar plates containing 2% horse blood and incubated overnight at 37 °C. Swabs giving one or more GAS colony-forming units were considered positive. Statistical significance between groups was determined by the Mantel log-(rank) test for survival curve analysis and by the χ^2 test for throat swab status, with $p < 0.05$ taken as statistically significant. Statistical analysis of antibody titers between groups was performed using a two-way ANOVA followed by the Bonferroni post hoc test. GraphPad Prism 5 software was used for statistical analysis, with $p < 0.05$ taken as statistically significant.

Circular Dichroism. CD spectra were collected between 200 and 240 nm using a Jasco J-710 spectrophotometer. Samples (0.2 mg/mL peptide solutions in PBS) were measured at room temperature using quartz cuvettes (0.1 cm path length) continuously purged with nitrogen. Measurements were performed in triplicate scans with the average subtracted from the blank PBS solution. The mean residual ellipticity was calculated as published elsewhere[22] and plotted against the corresponding wavelengths.

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