Toward the Development of a Synthetic Group A Streptococcal Vaccine of High Purity and Broad Protective Coverage

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Using native chemical ligation, we synthesized a group A streptococcal (GAS) vaccine that contained three different GAS M protein peptide epitopes in a chemically well-characterized construct in high purity. Two of the peptide epitopes represented variable amino terminal serotype determinants, and the third represented a carboxyl terminal conserved region determinant of the GAS M protein. We also synthesized a lipid core peptide (LCP) construct containing the same three peptides. Upon immunization of mice, the non-LCP construct only elicited antibody responses to all three epitopes with the use of adjuvant. The LCP construct, however, elicited excellent antibody responses to all three epitopes without the need for any additional adjuvant or carrier. We have synthesized the LCP synthetic vaccine system with good reproducibility.

Introduction

Short synthetic peptides generally do not elicit effective antibody responses upon immunization with the individual peptide epitope sequences. In animal studies, synthetic peptides often need to be coupled to larger proteins, such as KLH or BSA, to provide the necessary T-cell help that is required to elicit strong antibody responses to the peptide epitope(s) of interest. Because of the potentially harmful biological side effects, this strategy is not generally acceptable for use in humans. Therefore, several approaches have been investigated to enhance the antigenicity of synthetic peptides for human use. The MAP system[1] is based on using a polylysine core onto which several copies of immunogenic peptides are synthesized. In the LCP system,[2] the carrier, adjuvant, and antigen are contained in the same molecular entity. Another approach is polymerization that results in copolymers of different epitopes.[3,4] The problem with this latter method, however, is the difficulty in purification and characterization of the final product. In the case of the MAP and LCP systems, the number of different peptide epitopes that can be incorporated into the constructs is limited, which can be problematic in developing a vaccine with broad strain coverage.

Chemical ligation[5−7] offers a different approach to the synthesis of synthetic peptide vaccines with high purity and activity. Rose and co-workers[8,9] synthesized a high-purity protein vaccine based on the formation of oxime bonds among the four aldehyde groups of a template and the amino oxyacetyl groups of the unprotected and purified peptide comprising the C-terminal 23 residues of influenza virus hemagglutinin. For comparison, a traditional MAP construct was synthesized containing four copies of the 23-residue peptide on a Lys-Lys(Lys)-resin. The polyoxime construct resulted in a product of higher purity with significantly greater levels of antibody production induced following immunization when compared with the traditional MAP construct.

In our previous research, we investigated the LCP system[10] as a potential vaccine design and delivery approach for GAS compounds. GAS compounds are human pathogens that cause a variety of illnesses and diseases, ranging from the relatively minor pharyngitis to more severe invasive disease and the poststreptococcal sequelae−rheumatic heart disease and acute glomerulonephritis.[11,12] The surface M protein is an important virulence factor during GAS infection, and it has been shown that opsonic antibodies directed to the M protein type-specific and conserved regions are important in the development of protective immunity.[13,14] In our previous studies, we showed that mice immunized with either a single or two different GAS peptide epitopes incorporated into the LCP system generated specific antibodies to the GAS peptide epitopes even in the absence of a conventional adjuvant.[10,15,16] They also evoked high levels of opsonic antibodies against the relevant serotype.[15,16] Furthermore, mice were completely protected following immunization with the LCP construct and challenge with the serotype GAS strain included in the construct.[16]

The objective of this study was to further develop a synthetic GAS vaccine with increased purity, reproducibility and protective potential.

Results and Discussion

We targeted two (8830, Ac-DNGKA IYERA RERAL QELGP; PL1, Ac-EVLTR RQSQD PKYVT QRIS) of seven common amino terminal serotype epitopes, the sequence of which were derived from GAS isolates obtained from Australian Aboriginal patients in the

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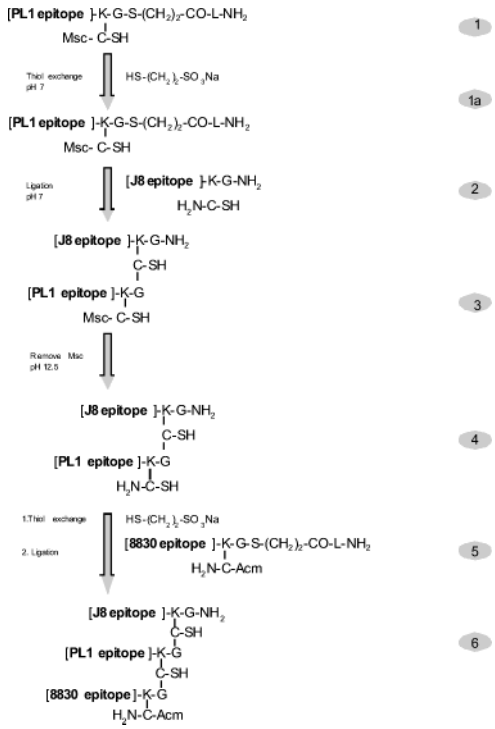

Figure 1. Synthesis of GAS vaccine via native chemical ligation of epitope fragments.

Northern Territory, a region highly endemic for GAS compounds.[4] We also included a conserved epitope (J8, Ac-QAEDK VKQSR EAKKQ VEKAL KQLED KVQ) in order to generate a vaccine with broader strain coverage. Such a combined vaccine approach is designed to provide increased protection against GAS infection by targeting both serotype and conserved region determinants of the GAS surface M protein.

Synthesis. The GAS vaccine was synthesized via native chemical ligation of the purified peptide epitope sequences (Figure 1). Epitopes **1** and **5** were synthesized on a Boc-Lys(Fmoc)-Gly-S-mercaptopropionyl-leucyl-MBHA resin, while epitope **2** was synthesized on a Boc-Lys(Fmoc)-Gly-MBHA resin using rapid *t*-Boc-amino acid/HBTU/DIPEA in situ neutralization chemistry.[17] On completion of the epitope synthesis, the Fmoc protection was removed from the ϵ-amino function of the lysine using the deblocking reagent of Li at al,[18] which efficiently removes the Fmoc group while keeping the thioester intact. An appropriately protected cysteine residue was coupled to the resulting free amino group. After HF cleavage and RP-HPLC purification, each peptide epitope was isolated in high purity. Mass spectrometry and analytical HPLC were used to characterize the individual peptides.

Ligation. The ligation steps (Figure 1) were carried out in pH 7 sodium phosphate buffer in the presence of mercaptoethane as a thiol additive. The transesterification of the mercaptopropionyl ester derivative **1** into the more reactive merchaptoethane derivative **1a** took place rapidly and quantitatively. The new ester derivative **1a** appearing as a new peak in the chromatogram was isolated and then characterized by mass spectrometry. Upon completion of the thiol exchange, the J8 derivative **2** was added to the reaction mixture and the formation of the J8−PL1 conjugate 3 was followed by analytical HPLC and mass spectrometry. When the

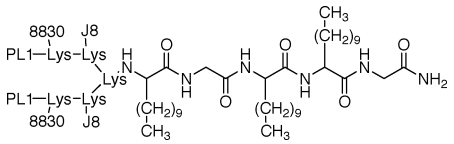

Figure 2. Structure of the LCP system.

ligation was completed, the Msc protecting group was removed in situ by a brief (5 min) treatment at pH 12.5[5] and the reaction mixture was purified by HPLC resulting in the pure conjugate **4**. The second ligation step was carried out similarly to the first one between the terminal Cys residue of the J8−PL1 conjugate **4** and the ester derivative of the 8830 epitope **5** resulting in the final GAS conjugate **6**.

Synthesis of the LCP System. An LCP construct containing three lipoamino acid C12-LAA residues, two glycine spacers, and two copies of the J8, 8830, and PL1 epitopes was synthesized on MBHA resin using Boc chemistry (Figure 2). A glycine spacer was employed between the MBHA resin and the first C12-LAA. Previous experiments indicated that higher antibody responses were achieved by incorporating glycine as a spacer between the second and third C12-LAA residues.[19] To introduce different peptide epitopes into the LCP system, orthogonally protected Boc-Lys(Fmoc)-OH was employed. The LCP construct was removed from the resin using hydrogen fluoride and characterized by SDS−PAGE and electrospray ionization mass spectrometry.

Immunization. Serum IgG antibody responses to the J8−8830−PL1 and LCP−J8−8830−PL1 GAS constructs were measured in groups of B10.BR mice immunized with the constructs in the presence or absence of CFA. For the J8−8830−PL1 construct given in CFA, antibody responses were detected in all mice to the J8, 8830, and PL1 GAS peptide epitopes, as early as 3 weeks after the initial primary immunization (Figure 3), with a final average titer of 1.45×10^6, 3.28×10^6, and 1.15×10^6, respectively. Positive controls were included for two of the peptides (J8/CFA and 8830/CFA), and these gave final average antibody titers of 5.12×10^5 and 1.88×10^6 to J8 and 8830, respectively. Antibody responses to the J8 and 8830 peptides in mice immunized with the construct in CFA were as good as those detected in mice immunized with the free peptides alone in CFA. Negative control mice immunized with CFA alone did not elicit antibody responses to any of the three peptides nor did mice immunized with the J8−8830−PL1 construct in the absence of adjuvant or when admixed with LCP (Figure 3).

For the LCP−J8−8830−PL1 construct (Figure 4) given in CFA, antibody responses were detected in all mice to the J8, 8830, and PL1 GAS peptide epitopes with a final average titer of 2.43×10^5, 1.23×10^6, and 1.54×10^6 to the J8, 8830, and PL1 peptides, respectively. When given in the absence of adjuvant, the LCP−J8−8830−PL1 construct yielded average antibody titers of 5.31×10^4, 6.66×10^5, and 2.87×10^5 to the J8, 8830, and PL1 peptides, respectively. Antibody responses to the J8 and 8830 peptides in mice immunized with the construct in CFA were as good as those detected in mice immunized with the free peptides alone in CFA (for J8 + 8830/CFA-immunized mice average antibody titers were 8.67×10^5 and 1.88×10^5 to the J8 and 8830

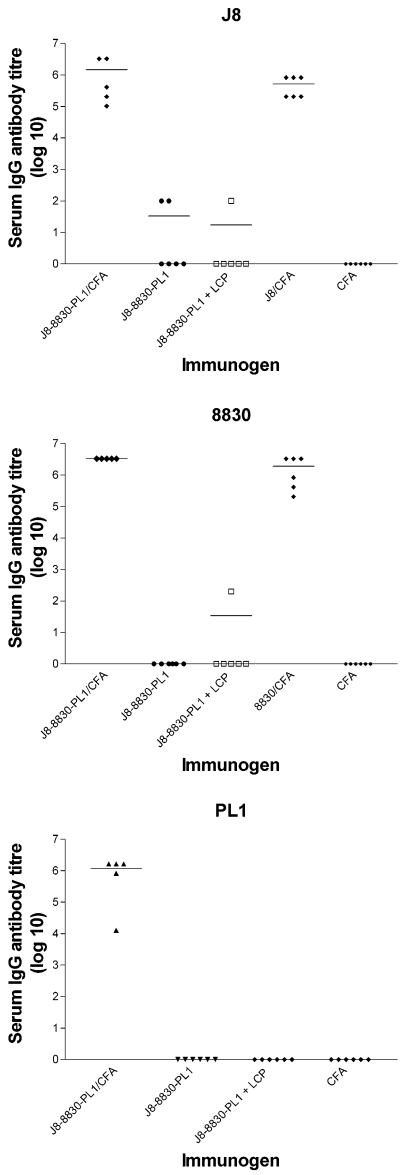

Figure 3. Serum IgG antibody response in B10.BR mice immunized parenterally with the J8−8830−PL1 GAS vaccine formulation in the presence and absence of CFA and admixed with LCP on day 49. Antibody titers to the J8, 8830, and PL1 GAS peptides for individual mice are shown with the average titer (arithmetic mean) represented as a bar. Antibody titers to the J8 and 8830 peptides are shown for individual control mice that were immunized with J8 peptide in CFA or with 8830 peptide in CFA, respectively. Mouse no. 5 in the J8−8830−PL1/CFA-immunized group died prior to analysis.

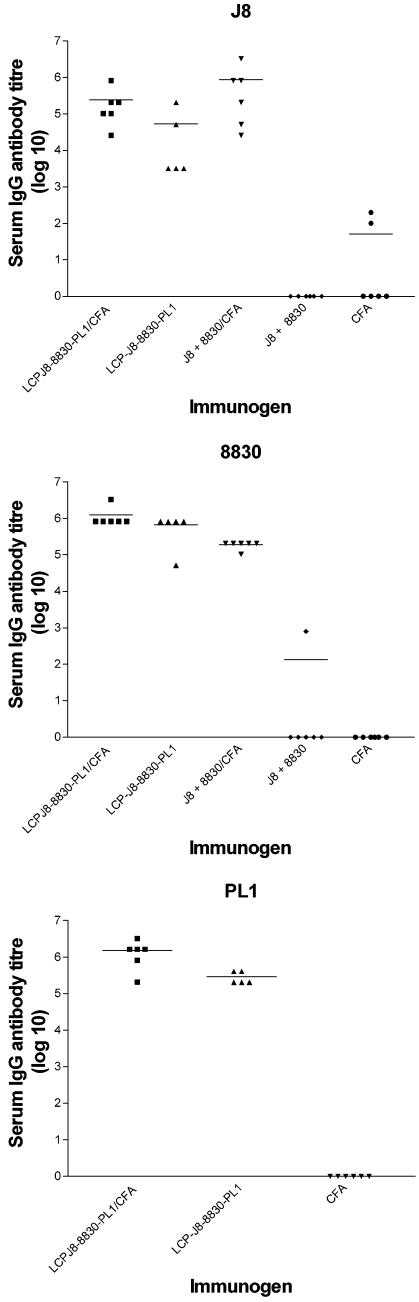

Figure 4. Serum IgG antibody response in B10.BR mice immunized parenterally with the LCP−J8−PL1−8830 GAS vaccine formulation in the presence and absence of CFA on day 49. Antibody titers to the J8, 8830, and PL1 GAS peptides for individual mice are shown with the average titer (arithmetic mean) represented as a bar. Antibody titers to the J8 and 8830 peptides are shown for individual control mice that were immunized with J8 and 8830 peptides in the presence or absence of CFA. Mouse no. 6 in the LCP−J8−8830−PL1-immunized group died prior to analysis.

peptides, respectively). Negative control mice immunized with CFA alone did not elicit antibody responses to any of the three peptides nor did mice immunized with the J8 + 8830 peptides alone (Figure 4).

Conclusion

We have synthesized GAS vaccine candidates containing two variable serotype determinants and a conserved region peptide epitope of the GAS M protein in high purity. By use of native chemical ligation, the three peptides were coupled together and they were also synthesized as an LCP construct. Both of the constructs elicited antibody responses to all three epitopes when administered with CFA, but only the LCP complex gave good antibody responses without the use of any additional adjuvant or carrier. The LCP construct was synthesized with good reproducibility, opening the way to the production of a new human vaccine.

Experimental Section

Materials and Methods. Boc-L-amino acids and MBHA resin were purchased from Novabiochem (Läufelfingen, Switzerland). DMF, TFA, DIEA, DCM (all peptide synthesis grade) were purchased from Auspep (Melbourne, Australia), and HBTU was obtained from Richelieu Biotechnologies (Quebec,

Canada). Electrospray ionization MS was obtained on a Perkin-Elmer API 3000 instrument using acetonitrile−water as mobile phase. Peptides were prepared by manual solid-phase peptide synthesis on a 0.25 mmol scale by using in situ neutralization/HBTU activation procedure for Boc chemistry as described by Schnölzer et al.[17] Each synthetic cycle consisted of *N*-Boc removal by a 1−2 min treatment with neat TFA, a 1 min DMF flow wash, a 10−20 min coupling time with 4-fold excess of preactivated Boc-amino acid in the presence of excess DIEA, and a second DMF flow wash. *N*-Boc-amino acids were preactivated immediately before coupling with an equivalent amount of HBTU (0.5 M HBTU in DMF) in the presence of excess DIEA. After each coupling step, yields were determined by measuring residual free amine with the quantitative ninhydrin assay.[20] After coupling of Gln residues, a dichloromethane wash was used before and after deprotection by using TFA, to prevent possible high-temperature (TFA/DMF) catalyzed pyrrolidone formation.

Boc-amino acids were used with the following side chain protection: Arg(Tos), Asp(OcHxl), Asn and Gln unprotected, Glu(OcHxl), Cys(4-MeBzl), Lys(2ClZ), Lys(Fmoc), Ser(Bzl), Thr(Bzl) and Tyr(BrZ). Racemic LAA were synthesized according to Toth et al.,[21] and their Boc protection was carried out by standard methods.

After completion of the synthesis, the peptide was removed from the resin support with the high HF method (1 mL of cresol, 10 mL of HF) to yield the crude peptide, which was precipitated with ice-cold ethyl ether and redissolved in 50% acetonitrile in water (25 mL). The reaction mixture was lyophilized.

Peptide purification was done on a Waters HPLC system (model 600 controller, 490 E UV detector, 60 F pump) using a preparative Vydac protein and peptide C18 column (2 cm × 25 cm). HPLC grade acetonitrile and water were filtered through a membrane filter and degassed with helium prior to use. Separation was achieved with a solvent gradient beginning with 100% solution A (0.1% trifluoroacetic acid in water), increasing constantly to 90% solution B (90% acetonitrile, 10% water, and 0.1% trifluoroacetic acid) for 40 min, and staying at this concentration for 10 min at a constant flow of 12 mL/min. Compounds were detected at 230 nm.

Analytical reversed-phase HPLC was performed on a Shimadzu instrument (LC-10AT liquid chromatograph, SCL-10A system controller, SPD-6A UV detector, and a SIL-6b autoinjector with an SCL-6B system controller) using a Vydac C-18 column (5 μm, 0.46 cm × 25 cm). Separation was achieved with solvent gradients of 0−100% solution B or 10−50% solution B for 30 min at a constant flow of 1 mL/min. Compounds were detected at 214 nm.

Another separation was achieved with solvent gradient of 0−100% solution B (90% methanol, 10% water, and 0.1% trifluoroacetic acid) for 30 min at a constant flow of 1 mL/min.

Native Chemical Ligation. The ligation of unprotected synthetic peptide epitopes was performed as follows: purified peptide thioester (8 μmol) was dissolved in 10 mL of 0.1 M phosphate buffer (pH 7), and an amount of 70 μmol of HS−CH_2−CH_2−SO_3Na crystals was added to the solution. It was kept at room temperature for 30 min. The thiol exchange was followed by analytical HPLC. In all cases it was complete after 30 min.

The purified epitope (8.2 μmol) containing unprotected Cys residue was dissolved in 10 mL of 0.1 M phosphate buffer (pH 7). Tris(2-carboxyethyl)phosphine (17 mg, 60 μmol) was added to the solution, and the pH was adjusted to 4.5. The mixture was kept at room temperature for 30 min. After this period the two peptide solutions were mixed, the pH was adjusted to 7, and the mixture was kept at room temperature. The ligation reaction was monitored by HPLC; at 2 h intervals a 20 μL sample of ligation mixture was injected into the HPLC to follow the consumption of the peptides and the formation of the ligated product. The content of the new peak was identified by electrospray ionization MS. After completion of the ligation (8−12 h), the Msc protecting group was removed by a 5 min treatment at pH 12.5. After that, the pH was adjusted to 7 and the mixture was loaded on a semipreparative HPLC column (Vydac C-18, 5 μm, 1 cm × 25 cm). Separation was achieved with a solvent gradient beginning with 15% solution B, increasing constantly to 40% solution B for 40 min at a constant flow of 4 mL/min.

Physical Data of the Purified Peptide Fragments and Ligated Products. Analytical HPLC retention times (t_{R1}, t_{R2} shown below) of the peptides were obtained by a solvent gradient beginning with 10% solvent B, increasing constantly to 50% solvent B for 30 min at a constant flow of 1 mL per min, versus 0−100% B, 30 min, 1 mL/min.

[1] Ac-[PL1 = EVLTR RQSQD PKYVT QRIS]-K(Msc-C-{SH})-G-S-$(CH_2)_2$-CO-L-NH_2: t_{R1} = 20.142 min (30% yield), t_{R2} = 24.292 min.

[2] Ac-[J8 = QAEDK VKQSR EAKKQ VEKAL KQLED KVQ]-K(C)-G-NH_{2L}: t_{R1} = 21.650 min (27.5% yield), t_{R2} = 26.700 min.

[3] Ac-[J8]-K{Ac-[PL1]-K(Msc-C)-G-C}-G-NH_2: t_{R1} = 22.658 min, t_{R2} = 26.500 min.

[4] Ac-[J8]-K{Ac-[PL1]-K(H_2N-C)-G-C}-G-NH_2: t_{R1} = 21.133 min (45% yield), t_{R2} = 25.232 min.

[5] Ac-[8830 = DNGKA IYERA RERAL QELGP]-K-(C{Acm})-G-S-$(CH_2)_2$-CO-L-NH_2: t_{R1} = 20.85 min (31% yield), t_{R2} = 25.545 min.

[6] Ac-[J8]-K(A)-G-NH_2 (A = Ac-[PL1]-K(C{B})-G-C-, B = Ac-[8830]-K(C{Acm})-G-C-): t_{R1} = 21.300 min (39% yield), t_{R2} = 26.992 min.

Chemistry of the LCP System. The LCP system was prepared by manual solid-phase peptide synthesis on a 0.25 mmol scale using MBHA resin (loading value: 0.54 mmol/g) and in situ neutralization/HBTU activation procedure for Boc chemistry. To synthesize different peptide epitopes within the same LCP construct, we introduced orthogonally protected lysines. The N-terminal group of each lysine was Boc-protected, and the ϵ-amino group was Fmoc-protected. By removing Boc protection, we achieved a free α-amine. Two copies of the J8 epitope were synthesized on these free amines using Boc chemistry while maintaining Fmoc protection of the lysine ϵ-amine groups. Following J8 synthesis, the Fmoc protecting groups were removed, and two copies of Boc-Lys-(Fmoc)-OH were coupled. Upon removal of the Boc protecting groups, two copies of the 8830 epitope were synthesized on the free amine groups. Followed by removal of the Fmoc-protecting groups, two copies of the PL1 epitope were synthesized on the free amine groups. After the synthesis of each peptide epitope, the free N-terminal amines were acetylated. Upon completion, the final product was removed from the resin by HF cleavage (1 mL of cresol, 10 mL of HF) and precipitated with ice-cold diethyl ether. The crude product was then redissolved in 50% acetonitrile/water (50 mL) and lyophilized.

To characterize the LCP construct, we used electrospray mass spectrometry (Perkin-Elmer API 3000, acetonitrile−water as the mobile phase) and SDS gel electrophoresis. MS of LCP multiply charged ions: $[M + 9H]^{9-}$, *m/z* 1918.1 (calcd 1918.5); $[M + 10H]^{10-}$, *m/z* 1725.8 (calcd 1726.8); $[M + 11H]^{11-}$, *m/z* 1569.2 (calcd 1569.9); $[M + 12H]^{12-}$, *m/z* 1438.2 (calcd 1439.1); $[M + 13H]^{13-}$, *m/z* 1329.2 (calcd 1328.5); $[M + 14H]^{14-}$, *m/z* 1233.1 (calcd 1233.7). SDS gel electrophoresis using Sigma myoglobin fragments as molecular weight markers showed a band around the 18 000 marker (calculated molecular weight of the LCP system was 17257.8).

The mass spectral data of the different peptide epitopes and ligation products are as follows.

1: $[M + 2H]^{2-}$, *m/z* 1493.1 (calcd 1493); $[M + 3H]^{3-}$, *m/z* 995.8 (calcd 995 0.7); $[M + 4H]^{4-}$, *m/z* 747.2 (calcd 747).

2: $[M + 3H]^{3-}$, *m/z* 1204.8 (calcd 1204.3); $[M + 4H]^{4-}$, *m/z* 904.6 (calcd 904.7); $[M + 5H]^{5-}$, *m/z* 723.4 (calcd 723.4); $[M + 6H]^{6-}$, *m/z* 602.9 (calcd 602.9).

3: $[M + 4H]^{4-}$, *m/z* 1595.8 (calcd 1595.8); $[M + 5H]^{5-}$, *m/z* 1276.8 (calcd 1276.9); $[M + 6H]^{6-}$, *m/z* 1064.6 (calcd 1064.5); $[M + 7H]^{7-}$, *m/z* 912.3 (calcd 912.5); $[M + 8H]^{8-}$, *m/z* 798.5 (calcd 798.4); $[M + 9H]^{9-}$, *m/z* 709.8 (calcd 709.9).

4: $[M + 4H]^{4-}$, *m/z* 1558.3 (calcd 1558.1); $[M + 5H]^{5-}$, *m/z* 1246.7 (calcd 1246.6); $[M + 6H]^{6-}$, *m/z* 1039.3 (calcd 1039); $[M + 7H]^{7-}$, *m/z* 890.8 (calcd 890.8); $[M + 8H]^{8-}$, *m/z* 779.4 (calcd 779.5); $[M + 9H]^{9-}$, *m/z* 693.2 (calcd 693).

5: $[M + 2H]^{2-}$, *m/z* 1445 (calcd 1445); $[M + 3H]^{3-}$, *m/z* 963.6 (calcd 963.7); $[M + 4H]^{4-}$, *m/z* 723.1 (calcd 723).

6: $[M + 6H]^{6-}$, *m/z* 1484.1(calcd 1483.5); $[M + 7H]^{7-}$, *m/z* 1272.2 (calcd 1271.7); $[M + 8H]^{8-}$, *m/z* 1113.3 (calcd 1112.9); $[M + 9H]^{9-}$, *m/z* 989.1 (calcd 989.4); $[M + 10H]^{10-}$, *m/z* 891 (calcd 890.5); $[M + 11H]^{11-}$, *m/z* 810.1 (calcd 809.7); $[M + 12H]^{12-}$, *m/z* 742.6 (calcd 742.3).

Mice and Immunization. For immunization, stock peptides were dissolved in sterile water at a concentration of 10 mg/mL and stored at −20 °C. Four to six week old female B10.BR mice (Animal Resource Centre, Perth, WA, Australia) were used ($n = 6$ per group) for subcutaneous immunization at the tail base with 30 μg of either J8−8830−PL1 or LCP−J8−8830−PL1 GAS construct, emulsified 1:1 with CFA (Sigma, Castle Hill, NSW, Australia) in a total volume of 50 μL sterile-filtered PBS or given alone in the absence of adjuvant. The J8−8830−PL1 GAS construct was also given admixed with LCP. Three weeks after the primary immunization, mice received a further four boosts at weekly intervals (i.e., days 21, 28, 35, and 42) with 3 μg of construct in PBS prior to the collection of blood on day 49. Controls received 30 μg of free peptide/s in the presence or absence CFA with boosts of 3 μg, or CFA alone.

Collection of Sera. During the course of the immunization protocol, 10 μL of blood was collected at various time points by tail snip and added to 90 μL of PBS. Following centrifugation at 3000 rpm for 10 min, sera were collected and stored at −20 °C. At the end of the immunization protocol (day 49), blood was collected from each mouse by the tail artery and allowed to clot at 37 °C for 1 h followed by the removal of clots by centrifugation at 3000 rpm for 10 min. Sera were then stored at −20 °C.

ELISA. Enzyme-linked immunosorbent assay was performed for the measurement of total IgG antibody titers in sera, essentially as previously described.[22] Sera samples were assayed using 2-fold dilutions of a 1 in 100 dilution of sera. Antibody titers were defined as the lowest dilution that gave an optical density (OD) reading at 450 nm of more than 3 standard deviations above the mean OD of control wells containing normal mouse sera (obtained from mice immunized with CFA only).

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Appendix

Abbreviations. GAS, group A streptococcal; LCP, lipid core peptides; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; MAP, multiple antigen peptides; MBHA, 4-methylbenzhydrylamine; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA or DIEA, *N*,*N*-diisopropylethylamine; Boc, *tert*-butoxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; HF, hydrofluoric acid; RP-HPLC, reverse-phased high-pressure liquid chromatography; C12-LAA, 2-aminododecanoic acid; CFA, complete Freund's adjuvant; MS, mass spectrometry; DMF, dimethylformamide; DCM, dichloromethane; TFA, trifluoroacetic acid; PBS, phosphate buffer solution; ELISA, enzyme-linked immunosorbent assay; SDS−PAGE, sodium dodecyl sulfate−polyacrilamide gel electrophoresis.

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