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Method for the Synthesis of Multi-Epitopic *Streptococcus pyogenes* Lipopeptide Vaccines Using Native Chemical Ligation

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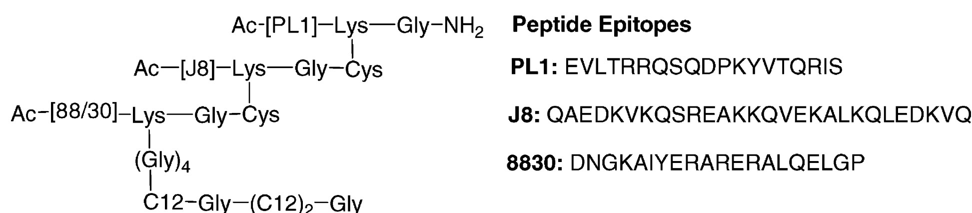

The aim of this study was to investigate methods for the synthesis of highly pure, well-characterized analogues of the lipid core peptide (LCP) system. Difficulties synthesizing and purifying conventional LCP systems have led to the requirement for a technique to produce highly pure, LCP-based vaccines for potential use in human clinical trials. The current study describes methods for the attachment of lipophilic adjuvants onto multi-epitopic peptide vaccines. Described is the synthesis, using native chemical ligation, of a highly pure, tri-epitopic, group A streptococcal (GAS) lipopeptide vaccine candidate. Intranasal immunization of the described tri-epitopic GAS lipopeptide with the mucosal adjuvant cholera toxin B subunit induced high serum IgG antibody titers specific for each of the incorporated peptide epitopes.

Introduction

Group A Streptococcus (GAS; *Streptococcus pyogenes*) is responsible for many diseases, including streptococcal toxic shock syndrome and necrotizing fasciitis, with streptococcal pharyngitis and impetigo being the most common GAS associated conditions. In a small number of GAS infections, patients who have not been treated, or treated inadequately, may develop acute rheumatic fever. Acute rheumatic fever (ARF) is an autoimmune disease characterized by the production of antibodies and T-cells against GAS, which cross-react with human tissues in the heart, joints, and brain.[1] The associated heart valve and pericardial inflammation may lead to rheumatic heart disease (RHD) and eventually heart failure. As ARF and RHD only occur following GAS infection, a means to prevent GAS infection (e.g., a vaccine) would provide the best opportunity to prevent these diseases.

Several prophylactic GAS vaccines are currently under development.[2−7] Many of these vaccines target the GAS M protein, an α-helical coiled-coil cell surface protein that is associated with resistance to phagocytosis.[1] The sequence of the GAS M protein amino (N)-terminus is highly variable between serotypes, with serotype-specific antibodies elicited to this region.[1] As over 100 GAS serotypes have been characterized, vaccines based on N-terminal peptides need to be multi-epitopic, including epitopes selected to offer broad-strain coverage against circulating serotypes. In comparison, the M protein carboxyl (C)-terminus region is highly conserved

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(1) Batzloff, M. R.; Sriprakash, K. S.; Good, M. F. *Curr. Drug Targets* **2004**, *5*, 57.
(2) Kotloff, K. L.; Corretti, M.; Palmer, K.; Campbell, J. D.; Reddish, M. A.; Hu, M. C.; Wasserman, S. S.; Dale, J. B. *JAMA* **2004**, *292*, 709.
(3) Hruby, D. E.; Hodges, W. M.; Wilson, E. M.; Franke, C. A.; Fischetti, V. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5714.
(4) Ji, Y. D.; Carlson, B.; Kondagunta, A.; Cleary, P. P. *Infect. Immun.* **1997**, *65*, 2080.
(5) Brandt, E. R.; Sriprakash, K. S.; Hobb, R. I.; Hayman, W. A.; Zeng, W.; Batzloff, M. R.; Jackson, D. C.; Good, M. F. *Nat. Med.* **2000**, *6*, 455.
(6) Olive, C.; Batzloff, M.; Horváth, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. *Infect. Immun.* **2003**, *71*, 2373.
(7) Horváth, A.; Olive, C.; Karpati, L.; Sun, H. K.; Good, M.; Toth, I. *J. Med. Chem.* **2004**, *47*, 4100.

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FIGURE 1. The lipid core peptide system.

between GAS serotypes,[1] with C-terminal peptide-based vaccines offering the potential to protect against multiple GAS serotypes.

The lipid core peptide (LCP) system[8] (Figure 1) represents a promising system for mucosal vaccine development. This system takes advantage of peptide lipidation, using synthetic lipo-amino acids,[9] and the poly-lysine multiple antigen peptide (MAP) system[10] to produce vaccines incorporating peptide antigens, a carrier, and an adjuvant into a single molecular entity. Many studies have demonstrated the capacity of LCP systems incorporating GAS antigens to elicit systemic IgG antibodies without the need for additional adjuvants when administered subcutaneously to mice[6,7,11] and in some cases following intranasal administration (personal communication, Colleen Olive). While the LCP system has many advantages as a vaccine delivery system, there are potential purification difficulties associated with their synthesis using stepwise solid phase peptide synthesis (SPPS), the use of a MAP system,[12] and the use of racemic lipo-amino acids.

Previously, we have published work toward the synthesis of a highly pure and highly characterized prophylactic GAS lipopeptide vaccine.[7] This study involved the synthesis of a peptide containing three GAS epitopes using native chemical ligation. Two of these epitopes (88/30: DNGKAIYERARERALQELGP and PL1: EVLTRRQSQDPKYVTQRIS)[5] were N-terminal peptides from GAS strains common to Australian Aboriginal populations of northern Queensland and the Northern Territory. The third epitope (J8: QAEDKVKQ**SREAKKQVEKAL**KQLEDKVQ)[13] was a conserved C-terminal peptide (in bold), flanked by sequences to maintain the conformational coiled-coil structure of the native M protein. Inclusion of J8, which includes a conserved C-terminal B cell epitope, offers the capacity to elicit antibodies which protect against multiple GAS serotypes. Since this vaccine did not contain the lipo-amino acid based built in adjuvant, it was not immunogenic

(8) Toth, I.; Danton, M.; Flinn, N.; Gibbons, W. A. *Tetrahedron Lett.* **1993**, *34*, 3925.
(9) Gibbons, W. A.; Hughes, R. A.; Charalambous, M.; Christodoulou, M.; Szeto, A.; Aulabaugh, A. E.; Mascagni, P.; Toth, I. *Liebigs Ann. Chem.* **1990**, 1175.
(10) Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5409.
(11) Olive, C.; Batzloff, M. R.; Horváth, A.; Wong, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. *Infect. Immun.* **2002**, *70*, 2734.
(12) Moore, M. L.; Grant, G. A. In *Synthetic Peptides, A User's Guide*, 2nd ed.; Grant, G. A., Ed.; Oxford University Press: New York, 2002; p 10.
(13) Hayman, W. A.; Brandt, E. R.; Relf, W. A.; Cooper, J.; Saul, A.; Good, M. F. *Int. Immunol.* **1997**, *9*, 1723.

SCHEME 1. Synthetic Approach to the Synthesis of 3

SCHEME 2. Synthetic Approach to the Synthesis of 6

without the addition of complete Freund's adjuvant (CFA) when administered subcutaneously.[7] As CFA is highly toxic and not compatible with human use, an aim of future studies was to investigate methods for the incorporation of the LCP lipidic adjuvant into this vaccine and similar structures.

The objective of this study was to develop methods, using native chemical ligation, for the development of highly pure, highly characterized GAS vaccines, incorporating the LCP lipidic adjuvant (Figure 1), as well as the previously described PL1, 88/30, and J8 peptides. Presented herein is the synthesis of branched peptides containing both a protected cysteine residue and C-terminal thioester compatible with native chemical ligation and optimization of their synthesis. These peptides contain one GAS epitope per peptide and are used as building blocks. A discussion of the methods tried for incorporating the LCP lipidic adjuvant into the vaccine using native chemical ligation is presented. A future aim of this research is to develop a mucosal prophylactic GAS vaccine. The capacity of the synthesized tri-epitopic vaccine to elicit antigen-specific systemic IgG antibodies when administered with a conventional mucosal adjuvant, cholera toxin B subunit (CTB), was therefore assessed.

Results and Discussion

Peptide building blocks Ac-J8-K[C(Acm)]-G-MPAL (**1**), Ac-PL1-K(C)-G-NH_2 (**2**), and Ac-88/30-K[G-C12-C12-G-C12-$(G)_4$]-G-MPAL (**5**) (Schemes 1 and 2) were synthesized to incorporate a N^{ϵ}-Fmoc-protected lysine residue, which following peptide epitope synthesis on the α-amine and acetylation of the terminal amine was deprotected and coupled to a cysteine residue [either acetamidomethyl (Acm) or *p*-methylbenzyl (*p*MeBzl) protected] to enable ligation of other peptide epitopes. Synthesis of these building blocks was achieved using a modified version of the Boc chemistry in situ neutralization protocol of Schnölzer et al.[14] This involved using 1 equiv of *N*,*N*-diisopropylethylamine (DIPEA) to the amount of amino

(14) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. *Int. J. Pept. Protein Res.* **1992**, *40*, 180.

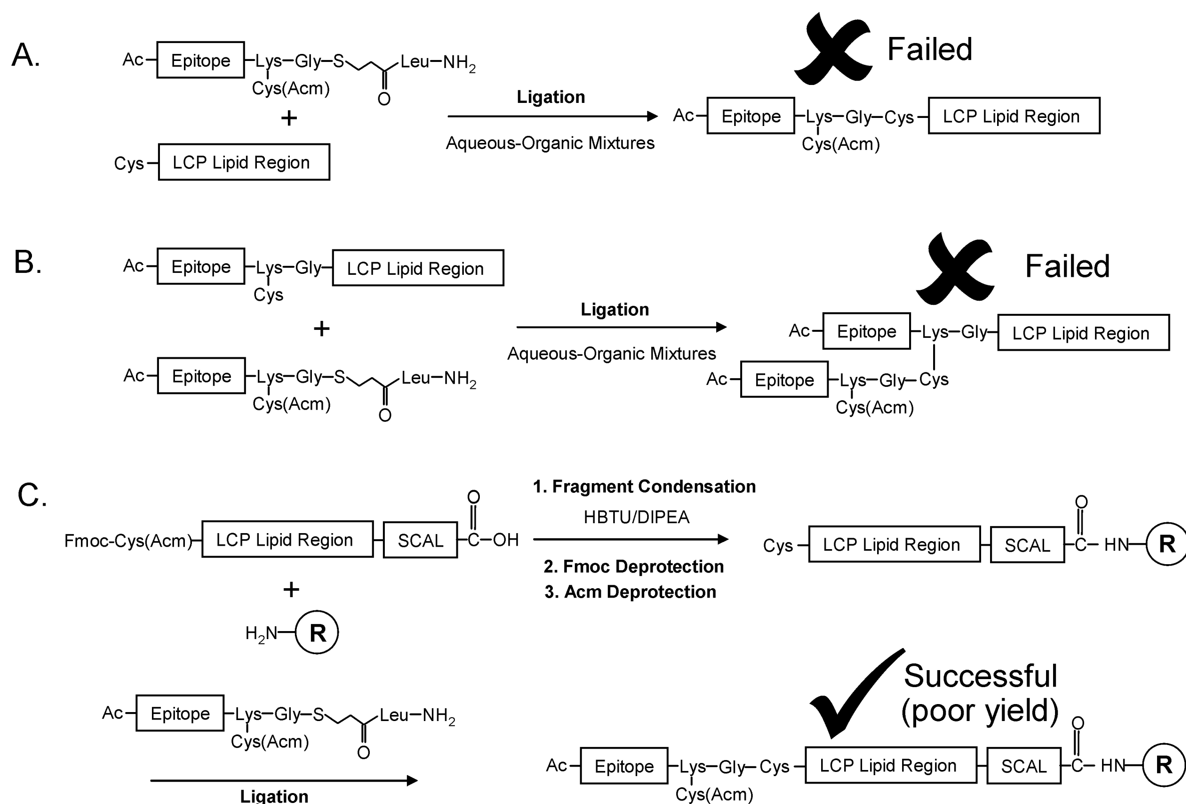

FIGURE 2. Attempted methods for conjugation of the LCP lipidic adjuvant and peptide epitopes. The safety catch acid labile linker (SCAL) of Pátek et al.[18] is utilized to enable cleavage of ligated peptides from the resin in example C.

acid, plus 1 equiv of DIPEA to the amount of resin, and an additional 30 μL of DIPEA. In comparison, the original manual protocol of Schnölzer et al. used 2 equiv of DIPEA to the amount of resin. This modification was utilized to reduce gradual lysine N^{ϵ}-Fmoc deprotection throughout the synthesis due to the presence of excess base, which was found to result in the formation of highly heterogeneous peptide mixtures when standard conditions were utilized. It was also found that, as the peptide chain increased in length, the lysine N^{ϵ}-Fmoc protecting group was stabilized. When Fmoc removal was required, this stabilization necessitated prolonged Fmoc deprotection times with monitoring by UV spectroscopic techniques. Fmoc deprotection conditions utilized for peptide **2** were 20% piperidine in DMF. However, piperidine could not be used for Fmoc deprotection of C-terminal thioester peptides **1** and **5** due to aminolysis of the thioester. Fmoc deprotection in the presence of a thioester was achieved using the method of Li et al.[15] This involved treating the resin with a mixture comprising 25% (v/v) 1-methylpyrrolidine, 2% (v/v) hexamethyleneimine, and 2% (w/v) 1-hydroxybenzotriazole (HOBt) in 1:1 1-methyl-2-pyrrolidinone (NMP):DMSO. This solution is capable of cleaving the Fmoc group without significant aminolysis of the C-terminal thioester.[15] Alternatively, the method of Clippingdale et al.[16] using the non-nucleophilic base DBU [1% (v/v)] together with 1% (w/v) HOBt in DMF was suitable for Fmoc deprotection in the presence of a C-terminal thioester.

(15) Li, X.; Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **1998**, *39*, 8669.
(16) Clippingdale, A. B.; Barrow, C. J.; Wade, J. D. *J. Peptide Sci.* **2000**, *6*, 225.

Native chemical ligation of peptide building blocks (**1**, **2**, and **5**) was performed in 0.1 M phosphate buffer, pH 7.6, containing sodium 2-mercaptoethanesulfonate (MESNA) as a thiol additive and tris-2-carboxyethylphosphine hydrochloride (TCEP) to prevent disulfide bond formation. These conditions enabled successful ligation of **1** and **2** (Scheme 1) to give the S-Acm-protected PL1-J8 di-epitopic peptide **3** in 65.3% following purification. However, this technique was not successful for ligation of **5** to the S-Acm-deprotected PL1-J8 di-epitopic peptide **4** due to solubility problems, resulting in the precipitation of **5**. Previously, we have published the synthesis of a tri-epitopic GAS peptide vaccine, which did not contain the LCP lipidic adjuvant, using these conditions.[7] The current study therefore aimed to use the same GAS epitopes to synthesize a highly pure vaccine incorporating the LCP lipidic adjuvant. Initial attempts to incorporate the LCP lipidic adjuvant into the vaccine using native chemical ligation investigated the use of organic solvent [2,2,2-trifluoroethanol (TFE), acetonitrile (ACN), DMF, DMSO, or dioxane] and phosphate buffer mixtures to ligate either a N-terminal cysteine-modified LCP lipidic adjuvant to a C-terminal thioester-modified GAS epitope (Figure 2A) or a lipopeptide featuring the LCP lipidic adjuvant to another GAS epitope (Figure 2B). These mixtures were initially found to solubilize peptides in the ligation reaction. However, within an hour, peptide precipitation was observed. This corresponded with ESI-MS and reversed phase high performance liquid chromatography (RP-HPLC) data, demonstrating that no ligation product formed (data not shown). An alternative approach to solve this problem involved fragment condensation of a N-terminal cysteine-modified analogue of the LCP lipidic adjuvant onto a water-compatible resin, followed by ligation of GAS

epitopes (Figure 2C) using the method of Brik et al.[17] While this technique was successful, it required the use of an expensive linker[18] to enable peptide cleavage from the resin, and ligation efficiency was poor, with ligation reactions only reaching 30% completion when 1.5 equiv of thioester peptide was utilized (data not shown). These poor results may be partially attributed to the use of Acm cysteine protection, with mercury(II) acetate utilized for deprotection. While mercury(II) acetate was successfully utilized for Acm deprotection of **3** to give **4** in 63.4% yield, recent experiments (unpublished data, Peter Moyle) have demonstrated that the LCP lipidic adjuvant as well as the dendrimeric structure of these vaccines may cause difficulties precipitating mercury from the formed mercapto−peptide complex using thiols, such as β-mercaptoethanol (BME) or dithiothreitol. The use of Ag(I) salts has proven unsuccessful for Acm deprotection of these compounds in solution. However, the use of I_2 in 50% aqueous acetic acid (AcOH), followed by quenching with ascorbic acid and reduction of any formed disulfide bonds with TCEP, has been demonstrated to enable fast Acm deprotection and access to reduced cysteine residues.

Due to the difficulties associated with solid phase native chemical ligation and the use of organic−phosphate buffer mixtures, the surfactant sodium dodecyl sulfate (SDS) was tried using the conditions stipulated by Valiyaveetil et al.[19] SDS was capable of keeping the lipopeptide in solution throughout the ligation reaction and enabled successful ligation of di-epitopic peptide **4** and lipopeptide **5** (Scheme 2). This was most likely due to solubilization of the lipidic region of lipopeptide **5** within SDS micelles. In addition, it has recently been demonstrated that the use of MESNA, instead of the commonly utilized thiol additive thiophenol, may be advantageous when SDS is utilized to solubilize hydrophobic C-terminal thioester peptides.[20] The reason proposed for this is that MESNA is more likely to be oriented outside of micelles due to its hydrophilic nature, while thiophenol, due to its more hydrophobic nature, is likely to be buried inside SDS micelles. Accessibility of the thioester for nucleophilic attack by cysteine thiols is therefore facilitated to a greater extent by the use of MESNA. However, use of SDS causes its own problems. These problems include band broadening during RP-HPLC purification, sample signal suppression in the mass spectrometer, and peptide denaturation. While the use of SDS made purification of **6** more difficult, it could be successfully purified, in 41.3% yield, by preparative RP-HPLC when a C4 column was utilized.

Serum IgG antibody responses against each of the epitopes included in the vaccine were measured in immunized mice using an enzyme-linked immunosorbent assay (ELISA). Elicitation of mucosal sIgA antibodies was not assessed as they are unable to promote phagocytic killing of systemic GAS infections, although they have been demonstrated to offer protection against nasopharyngeal colonization.[21] The mice were administered lipopeptide vaccine **6** intranasally with CTB as a mucosal adjuvant. Strong serum IgG antibody titers were elicited to each of the included antigens at day 42 (Figure 3), which were comparable in magnitude to those observed previously with the subcutaneous administration of LCP systems containing the same epitopes, administered with or without CFA.[7] In addition, previous studies have demonstrated that an 88/30-containing LCP system, which elicited systemic 88/30-specific IgG antibody titers of 1.04×10^5 and 1.39×10^6 in separate experiments, was capable of protecting immunized mice against intra-peritoneal challenge with 88/30 GAS.[6] These data therefore suggest that the systemic 88/30-specific IgG antibodies elicited following intranasal administration of **6** may provide protection against 88/30 GAS infection.

FIGURE 3. Antigen-specific serum IgG antibody titers (day 42) induced in mice immunized intranasally with **6** administered with CTB. Antibody titers are shown for individual mice to the J8, 88/30, and PL1 GAS peptide epitopes. The average titer (geometric mean) is represented as a bar.

Conclusion

The use of native chemical ligation for the synthesis of highly lipophilic peptides has proven particularly difficult due to the need for aqueous reaction conditions. The current study demonstrates the use of SDS for solubilization of lipophilic peptides for use in native chemical ligation. Use of this technique has enabled the synthesis of an experimental branched tri-epitopic GAS lipopeptide vaccine in high purity. Intranasal administration of this vaccine with CTB elicited high serum antigen-specific IgG antibody titers against each of the incorporated peptide epitopes. Overall, this research has provided a novel method for the synthesis of highly pure, multi-epitopic lipopeptide vaccines that could potentially be utilized in human clinical trials.

Experimental Section

Native Chemical Ligation of 1a and 2. 1 (26 mg, 6.7 μmol) was dissolved in 0.1 M phosphate buffer pH 7.6 (1 mL) to which MESNA (11 mg, 67 μmol) was added. The thiol exchange was left to proceed for 1 h to form peptide **1a**. **2** (19.4 mg, 7.36 μmol) was dissolved in 0.1 M phosphate buffer pH 7.6 (3.9 mL) to which TCEP (6.3 mg, 22 μmol) was added. This solution was then transferred into the vessel containing peptide **1a**. After 5 h, the product **3** was purified by semipreparative RP-HPLC on a Vydac C4 column (214TP1010, 10 μm, 10 × 250 mm) using a gradient of 10% solvent B to 60% solvent B (solvent A: 0.1% TFA/H_2O; solvent B: 90% ACN/0.1% TFA/H_2O) over 45 min at a constant 4 mL/min flowrate. The fractions were analyzed by ESI-MS and,

(17) Brik, A.; Keinan, E.; Dawson, P. E. *J. Org. Chem.* **2000**, *65*, 3829.
(18) Pátek, M.; Lebl, M. *Tetrahedron Lett.* **1991**, *32*, 3891.
(19) Valiyaveetil, F. I.; MacKinnon, R.; Muir, T. W. *J. Am. Chem. Soc.* **2002**, *124*, 9113.
(20) Sato, T.; Saito, Y.; Aimoto, S. *J. Peptide Sci.* **2005**, *11*, 410.
(21) Bessen, D.; Fischetti, V. A. *Infect. Immun.* **1988**, *56*, 2666.

where appropriate, combined to give **3** (27.5 mg, 65.3% yield) following lyophilization.

S-Acm Deprotection of 3. Hg(II)$(OAc)_2$ (16 mg, 50 μmol) was dissolved in AcOH/H_2O solution, pH 4 (2.5 mL), and added to **3** (27.5 mg, 4.36 μmol). After 60 min, BME (0.2 mL, 2.9 mmol) was added and the reaction left for 5 h. The mixture was then loaded onto a 2 cm $\times$ 6 cm (20 mL) Sephadex G-25 medium column and eluted with acidified H_2O (pH 4, AcOH). The fractions containing **4** (by analytical RP-HPLC) were combined and lyophilized to give **4** (17.2 mg, 63.3% yield).

Native Chemical Ligation of 5a and 4. **5** (11.6 mg, 3.17 μmol) was dissolved in H_2O (3 mL) containing SDS (30 mg), frozen, and lyophilized. The powder was then rehydrated with 0.1 M phosphate buffer pH 7.6 (1 mL) to which MESNA (5.2 mg, 32 μmol) was added. Thioester exchange was left to proceed for 1 h to give **5a**. **4** (17.2 mg, 2.76 μmol) was dissolved in 0.1 M phosphate buffer pH 7.6 (2 mL) to which TCEP (4.75 mg, 16.6 μmol) was added. After 30 min, the pH was adjusted to 7.6 with 1 M NaOH solution (60 μL), and the solution was transferred into the vessel containing **5a**. After 48 h, the ligation mixture was diluted with 1:1 TFE/H_2O (7 mL). The product **6** was then purified by semipreparative RP-HPLC on a Vydac C4 column (214TP1010, 10 μm, 10 $\times$ 250 mm) using a gradient of 0% solvent B to 70% solvent B (solvent A: 0.1% TFA/H_2O; solvent B: 90% ACN/0.1% TFA/H_2O) over 60 min at a constant 4 mL/min flowrate. The fractions were analyzed by ESI-MS and, where appropriate, combined to give **6** (11.0 mg, 41.3% yield) following lyophilization.

Mice and Intranasal Immunization. All protocols were approved by the Bancroft Centre Research Animal Ethics Committee (approval number P415). Immunizations were conducted in 4−6 week old female B10.BR mice (H-2^k). Prior to intranasal immunization, the mice were anesthetized using isofluorane vapor. Mice ($n = 10$/group) then received 30 μg of vaccine **6** intranasally on day 0 with 10 μg CTB in a total volume of 20 μL sterile-filtered phosphate buffered saline (10 μL per nare). Controls received CTB or PBS. Mice received five boosts at weekly intervals (days 7, 14, 21, 28, and 35) prior to collection of blood on day 42 for ELISA.

Collection of Sera. Blood was collected from the tail artery of each mouse 1 week after the last 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 was then stored at −20 °C.

Detection of Systemic IgG Antibodies by ELISA. ELISA for determination of serum IgG antibodies against the PL1, 88/30, and J8 epitopes included in the vaccine was performed as previously described.[22] Briefly, serial dilutions of sera were produced in 0.5% skim milk PBS/tween 20 buffer, starting at 1:100 concentration with 2-fold dilutions. Antibody titers were assessed following the addition of peroxidase-conjugated goat anti-mouse IgG, and *O*-phenylenediamine. Optical density was read at 450 nm in a microplate reader. The antibody titer was defined as the lowest dilution with an optical density more than three standard deviations greater than the mean absorbance of control wells containing normal mouse serum.

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Supporting Information Available: Synthesis of peptides **1**, **2**, and **5**, analytical data, HPLC chromatograms, ESI-MS, and SDS−PAGE data are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) Pruksakorn, S.; Galbraith, A.; Houghten, R. A.; Good, M. F. *J. Immunol.* **1992**, *149*, 2729.