Lipoamino Acid-Based Adjuvant Carrier System: Enhanced Immunogenicity of Group A Streptococcal Peptide Epitopes

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Lipoamino acid-based synthetic peptides (lipid core peptides, LCP) derived from the type-specific and conserved region determinants of group A streptococci (GAS) were evaluated as potential candidate sequences in a vaccine to prevent GAS-associated diseases, including rheumatic heart disease and poststreptococcal acute glomerulonephritis. The LCP peptides had significantly enhanced immunogenicity as compared with the monomeric peptide epitopes. Furthermore, the peptides incorporated into the LCP system generated epitope-specific antibodies without the use of any conventional adjuvant.

Introduction

Small peptide molecules are usually not of sufficient molecular weight to be immunogenic but can be rendered immunogenic or more immunogenic by conjugation to a carrier molecule, for example, a protein or a synthetic polymer. Proteins such as bovine serum albumin, keyhole limpet haemocyanin, or diphtheria toxoid are widely used for this purpose. Although the conjugated product is antigenic, it comprises a large number of epitopes other than those associated with the synthetic peptide of interest. Di Marchi et al.[1] described a synthetic peptide comprising two immunologically significant regions of a virus coat protein joined by a spacer. The resulting peptide had adequate molecular weight to induce an immune response. However, the spacer region itself can potentially act as an antigenic epitope. In 1983, a synthetic analogue of the N-terminal moiety of bacterial lipoprotein from *Escherischia coli* (tripalmitoyl-*S*-glyceryl cysteine), termed Pam_3Cys, was described.[2] This construct was conjugated to B and T cell epitopes and after administration to animals generated long-lasting antibody and T helper responses[3] and cytotoxic T lymphocytes.[4] High antibody titers have been attained by coupling immunogenic peptides to a polylysine core, to form a multiple antigenic peptide.[5]

We have further refined this approach, by using a lipoamino acid (LAA)-based lipidic anchor moiety at the C terminus of the polylysine system (lipid core peptides, LCP).[6] In the LCP system, the carrier, adjuvant, and antigen are contained in the same molecular entity. This molecular entity can be readily synthesized in a single reaction vessel, by stepwise solid phase methods. Properties such as molecular weight, charge, lipophilicity, targeting moieties, radiolabels, and so forth can readily be varied in the LCP system. Promising immunological data were obtained in preliminary studies using the LCP system via the parenteral route; for example, the immunogenicity was found to be much greater for synthetic peptides from foot and mouth disease virus[7] incorporated into the LCP system than when synthetic peptide was combined with complete Freund's adjuvant (CFA).[8] Synthetic peptides derived from the variable domains of *Chlamydia trachomatis* outer membrane protein induced titers up to 3200-fold higher when in the LCP system, than when used with CFA.[9]

This study has investigated the LCP system as a self-adjuvanting antigen delivery system for the delivery of group A streptococcal (GAS) vaccine candidates based on the M protein. GAS are human pathogens that cause a variety of illnesses and diseases, ranging from the relatively minor pharyngitis to more severe, invasive diseases, the poststreptococcal sequelae-rheumatic heart disease and acute glomerulonephritis.[10−12] These diseases are a major health concern in developing countries and indigenous populations worldwide but especially in the Australian Aboriginal population.[12−14] Current vaccine strategies aimed to prevent GAS infection, and consequently GAS-associated diseases, have focused on the bacterial surface M protein. The M protein is composed of a highly variable amino terminal region, which defines the GAS serotype (there are at least 100 different GAS serovars), a series of A repeats and B repeats, and the C repeat region, which is highly conserved between different GAS strains.[11] The 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 region are important in the development of protective immunity.[15−17] Previous studies have demonstrated the protective potential of various M protein-based GAS vaccine candidates using an animal model of GAS infection, at both the systemic[17−19] and mucosal level.[20−22] The delivery of the vaccine candidates, however, relied on coadministration with adjuvants, such as CFA and cholera toxin B subunit, that are too toxic for human

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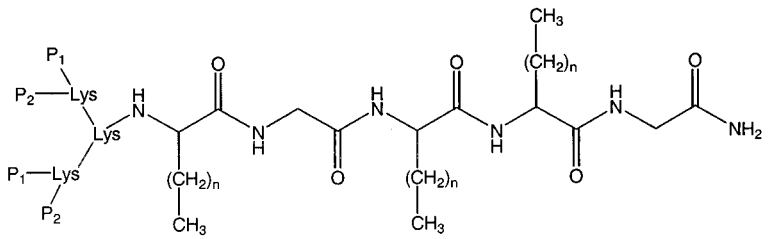

Figure 1. LCP system. LCP1: $P_1 = P_2$ = QAEDKVKQSREAKKQVEKALKQLEDKVQ; $n = 9$. LCP2: P_1 = QAEDKVKQSREAKKQVEKALKQLEDKVQ; P_2 = DNGKAIYERARERALQELGPC; $n = 7$.

use. Alternative vaccine design and delivery strategies must therefore be investigated to aid the progression of GAS vaccine research and development, with the eventual application to the clinic.

In this study, LCP constructs were synthesized incorporating peptides from either the M protein N terminus type-specific region and/or the conserved C terminus C repeat region. The constructs were used to immunize mice by the parenteral route prior to the assessment of immunogenicity.

Results and Discussion

The peptide epitopes J8 and 8830 were incorporated into the LCP system. The lipophilic anchor was constructed using three copies of racemic LAAs resulting in a diastereomeric mixture consisting of four pairs of enantiomers. A glycine was employed as a spacer between the MBHA resin and the first LAA. Our previous experiments (unpublished results) indicated that higher antibody responses could be achieved using another glycine spacer molecule between the second and third LAAs. An LCP construct (Figure 1), LCP1 (eight diastereomers), was synthesized containing three 2-amino-dodecanoic acids with the glycine spacers and four copies of epitope J8, using Boc synthesis methods. Another construct (LCP2) was synthesized containing three copies of 2-amino-decanoic acids with the above-described glycine spacers, two copies of epitope J8, and two copies of epitope 8830. The final compound was again a diastereomeric mixture of eight optically active compounds. The rationale for using two carbon shorter LAAs was to offset the slightly higher lipophilicity of epitope 8830, as compared with epitope J8. To introduce different peptide epitopes into the same LCP construct, orthogonally protected lysines were employed. The terminal amino group of each lysine was Boc-protected, while the ϵ-amino group was Fmoc-protected. Partial amino deprotection was achieved by removing the Boc protection initially. Peptide J8 was constructed on these amino groups, using Boc synthesis methods, while the other two amino functions of the lysines remained Fmoc-protected. On completion of the J8 synthesis, the two Fmoc protections were removed from the amino functions of the lysines and the synthesis of two copies of peptide 8830 was accomplished using standard Boc–amino acid coupling and deprotection methods.[24] The final cleavage using hydrogen fluoride resulted in the LCP peptide, LCP2.

Serum IgG Antibody Responses in B10.BR Mice Immunized with LCP Constructs. LCP1. Serum IgG antibody responses to the peptide J8 were measured in groups of mice immunized with the LCP1 construct, either with or without CFA (Figure 2A). We have

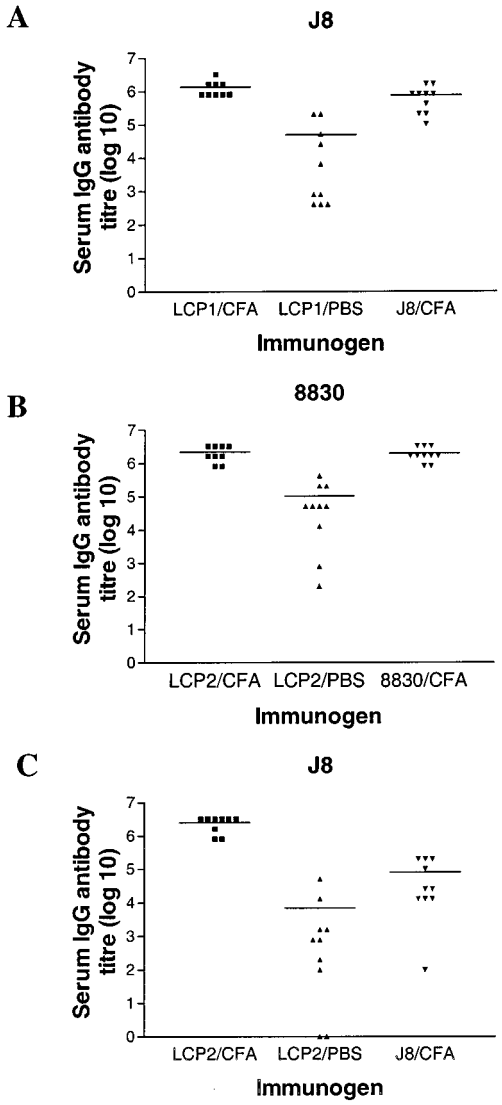

Figure 2. Serum IgG antibody responses in B10.BR mice immunized parenterally with the LCP1 (A) and LCP2 (B and C) constructs in the presence or absence of CFA, on day 49. For the LCP1 construct, antibody titers to the J8 peptide for individual mice are shown, with the average titer (arithmetic mean) represented as a bar. For the LCP2 construct, antibody titers to the 8830 and J8 peptides for individual mice are shown, with the average titer represented as a bar. Antibody titers are shown for individual control mice that were immunized with J8/CFA and 8830/CFA, to the J8 and 8830 peptides, respectively.

previously shown that the J8 peptide is not immunogenic in the absence of adjuvant after primary immunization followed by boostings with the free peptide. J8 was administered in CFA as a positive control. Three weeks after the primary immunization, J8-specific antibodies were detected in all mice immunized with

LCP1 in CFA and J8 in CFA, giving a final average antibody titer after 5 boosts of 1 365 333 and 747 520, respectively. J8-specific antibodies were not detected at 3 weeks postimmunization in mice immunized with LCP1 without adjuvant. However, after 1 boost of immunogen, 2 of the 10 mice had J8 antibodies, and after boost 3, J8 antibodies were detected in all mice. After the final boost (boost 5) the average J8 antibody titer in mice immunized with the LCP1 construct without adjuvant was 49 560. We have shown that the level of antibodies induced by immunization with the LCP1 construct in the absence of adjuvant is sufficient to impart protective immunity to GAS infection (unpublished results).

LCP2. High titer antibody responses were detected in all mice immunized with LCP2 in CFA to both J8 and 8830 GAS peptides (Figure 2B,C), as early as 3 weeks postimmunization, with a final average titer of 2 548 622 and 2 184 533, respectively. Positive controls (8830/CFA and J8/CFA) also gave high antibody titers of 1 966 080 and 89 600, respectively. Thus, the antibody responses to the 8830 peptide were similar in mice immunized with the free peptide alone in CFA and those immunized with LCP2 in CFA; antibody responses to the J8 peptide, however, were almost 3-fold higher in mice immunized with LCP2 in CFA as compared to those immunized with free peptide alone in CFA. J8 and 8830 peptide-specific antibodies were first detected in mice immunized with LCP2 without adjuvant after the first boost and gave a final average titer after the fourth boost of 6910 and 103 780, respectively.

Conclusion

We have previously demonstrated complete protection of mice from systemic GAS infection following immunization with an M protein-based multiepitope vaccine construct.[17] The efficacy of the construct, however, required administration in CFA, which is not suitable for human use and was delivered subcutaneously. This current study has investigated the LCP system as an alternative delivery strategy for a GAS vaccine and has demonstrated the immunogenicity of two different GAS-based LCP constructs in mice. Moreover, our data demonstrated the immunogenicity of these LCP constructs in the absence of any additional adjuvant. These findings indicate the potential use of the LCP system in the delivery of a synthetic GAS vaccine with self-adjuvanting properties, with a view to the development of a mucosal-based vaccine for human application.

Experimental Section

Chemistry. The peptide synthesis was carried out on solid phase using standard Boc chemistry.[23] After each coupling step, yields were determined by measuring residual free amine with the quantitative ninhydrin assay.[24] When necessary, further couplings were performed until the coupling yield reached a minimum of 99.8%. The LAAs were synthesized as described previously.[25] Amino acid analysis was carried out according to Cohen et al.[26] The details of the chemistry and the characterization of LCP1 and LCP2 are provided as Supporting Information.

Mice and Immunization. For immunization purposes, stock peptides were dissolved in sterile phosphate-buffered saline (PBS) 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 = 10 per group) for subcutaneous immunization at the tail base with 30 μg of LCP construct, either emulsified 1:1 with CFA (Sigma, Castle Hill, NSW, Australia) or given alone in a total volume of 50 μL of sterile-filtered PBS. After 3 weeks, mice received a further 4 boosts at weekly intervals with 3 μg of LCP construct in PBS prior to the collection of blood on day 49. Controls received 30 μg of free peptide in CFA with boosts of 3 μg.

Collection of Sera. 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.[27] 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 in PBS only).

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Supporting Information Available: Synthesis, purification, and characterization of compounds LCP1 and LCP2. This information is available free of charge via the Internet at http://pubs.acs.org.

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