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Potential of Lipid Core Peptide Technology as a Novel Self-Adjuvanting Vaccine Delivery System for Multiple Different Synthetic Peptide Immunogens

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This study demonstrates the effectiveness of a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens by use of lipid core peptide (LCP) technology. An LCP formulation incorporating two different protective epitopes of the surface antiphagocytic M protein of group A streptococci (GAS)—the causative agents of rheumatic fever and subsequent rheumatic heart disease—was tested in a murine parenteral immunization and GAS challenge model. Mice were immunized with the LCP-GAS formulation, which contains an M protein amino-terminal type-specific peptide sequence (8830) in combination with a conserved non-host-cross-reactive carboxy-terminal C-region peptide sequence (J8) of the M protein. Our data demonstrated immunogenicity of the LCP-8830-J8 formulation in B10.BR mice when coadministered in complete Freund's adjuvant and in the absence of a conventional adjuvant. In both cases, immunization led to induction of high-titer GAS peptide-specific serum immunoglobulin G antibody responses and induction of highly opsonic antibodies that did not cross-react with human heart tissue proteins. Moreover, mice were completely protected from GAS infection when immunized with LCP-8830-J8 in the presence or absence of a conventional adjuvant. Mice were not protected, however, following immunization with an LCP formulation containing a control peptide from a *Schistosoma* sp. These data support the potential of LCP technology in the development of novel self-adjuvanting multi-antigen component vaccines and point to the potential application of this system in the development of human vaccines against infectious diseases.

To induce effective immunostimulation and protective immunity, vaccines comprising a particular antigen or fragment thereof require a suitable adjuvant in addition to a carrier system. This is a critical issue with newer-generation vaccines such as subunit, recombinant, and synthetic vaccines, which, despite containing purer antigens, tend to be poorly immunogenic compared to live attenuated vaccine formulations (3, 43). The efficacy of conventional vaccine formulations, administered parenterally and mucosally in experimental animal models, has required the use of adjuvants such as complete Freund's adjuvant (CFA) (7) and cholera toxin (20), respectively, which are not suitable for use in humans due to their toxicity. Current vaccine formulations licensed for human use mainly contain alum-based adjuvants (such as aluminum hydroxide or aluminum phosphate) (18). This limited choice of adjuvants for human vaccination reflects a compromise between a requirement for adjuvanticity and an acceptably low level of toxicity.

Development of novel human vaccine delivery strategies for both existing and reemerging infections faces significant hurdles, particularly with regard to development of safe, effective, nontoxic adjuvants, in addition to the number of antigens that can be included in any one formulation, as multiple antigens may be required for successful vaccination against certain pathogens to provide broad coverage. Research is now focused on the development of vaccine adjuvants with improved immunogenicity, reduced toxicity, universal efficacy, and the potential for delivery via other routes, such as mucosal delivery for vaccination against many pathogens that infect mucosal surfaces. Recent advances in vaccine immunology have included the development of sophisticated antigen delivery systems, especially those based on synthetic peptide immunogens (23, 33), and the development of alternative adjuvants for vaccine delivery, including genetically manipulated bacterial toxins, monophosphoryl lipid A, immunostimulatory complexes, proteosomes (outer membrane proteins of *Neisseria meningitidis*), live commensal bacteria and bacterial particles, and novel lipid-containing compounds (reviewed in references 3, 18, 25, and 33).

The development of novel synthetic adjuvants offers the possibility of vaccine delivery without the need for additional adjuvant. Synthetic lipid-based compounds using various lipopeptides (11, 24, 28, 39, 40) or, as described by Toth and colleagues (45), using lipid core peptide (LCP) technology provide an attractive alternative for vaccine delivery. The LCP system incorporates lipoamino acids coupled to a polylysine core containing up to two different antigenic peptides (45) (see Fig. 1) and is uniquely designed to incorporate antigen, carrier, and adjuvant in a single molecular entity. The LCP system essentially combines the multiple antigen peptide (MAP) and

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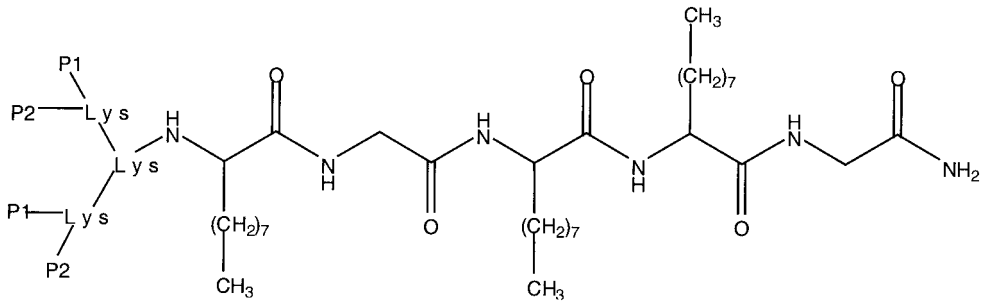

FIG. 1. Chemical structure of the LCP-8830-J8 (LCP-GAS) formulation. LCP-8830-J8 was synthesized containing three 2-amino-octanoic lipoamino acids (shown as branched alkyl side chains) attached to a polylysine core, with two copies each of the 8830 and J8 GAS peptides attached to the amino groups of each lysine. Glycine spacers were employed, one between the resin and the first lipoamino acid and one between the second and third lipoamino acids. In LCP-8830-J8, P1 stands for the 8830 GAS peptide (DNGKAIYERARERALQELGPC) and P2 stands for the J8 GAS peptide (QAEDKVKQ**SREAKKQVEKAL**KQLEDKVQ).

tripalmitoyl-*S*-glyceryl cysteine (Pam3Cys) systems. MAPs incorporate peptides coupled onto a polylysine core; significantly higher antibody titers have been obtained upon immunization with MAPs than by immunization with carrier protein-conjugated peptides in the presence of adjuvant (44). The Pam3Cys system utilizes a synthetic analog of the N-terminal moiety of bacterial lipoprotein from *Escherichia coli* as a lipid anchor moiety (26). When covalently linked to a peptide, tetrameric forms of a MAP, or polyoxime constructs, Pam3Cys lipopeptide compounds have been found to be potent immunogens with self-adjuvanting properties, eliciting humoral and cellular responses irrespective of the route of administration (27, 29, 30, 47). Most importantly, lipopeptides represent potentially safe vaccines for human application (1).

LCP-based vaccine candidates incorporating variable domains of *Chlamydia trachomatis* outer membrane protein have been shown to significantly enhance peptide immunogenicity over that of peptide monomers given alone in adjuvant (48), and an LCP compound incorporating a foot-and-mouth disease viral peptide was immunogenic, resulting in induction of anti-peptide antibodies in the absence of additional adjuvant (46). Recently, we investigated the LCP system as a vaccine delivery strategy for group A streptococci (GAS) (31), the causative agents of rheumatic fever (RF) and subsequent rheumatic heart disease (RHD) (4, 12). RF and RHD are a major health concern in developing countries and indigenous populations worldwide, but especially among Australian aboriginals, who suffer the highest disease rates (9). The bacterial surface anti-phagocytic M protein (12), a major GAS vaccine candidate, was the targeted antigen. Protective immunity to GAS infection has been associated with the presence of type-specific opsonic antibodies against M protein (12), although opsonic antibodies specific to the carboxy-terminal conserved C region have been demonstrated in humans (5) and mice immunized with C-region peptides (37) and are also important in eliciting protective immunity to GAS (6). Mice immunized parenterally, in the absence of a conventional adjuvant, with an LCP formulation containing a protective C-region determinant of the GAS M protein elicited high-titer, heterologous opsonic antibodies that did not cross-react with human heart tissue proteins (31), indicating the potential of such a vaccine for inducing broadly protective immune responses.

This study has further investigated the potential of LCP technology in the development of a novel self-adjuvanting multi-antigen component vaccine delivery system by using a murine model of parenteral immunization and GAS challenge. Mice were immunized with an LCP formulation incorporating two different protective epitopes of the GAS M protein—an amino-terminal type-specific peptide sequence (6) in conjunction with a conserved carboxy-terminal C-region peptide sequence (19) designed to lack a human heart-cross-reactive T-cell epitope (36)—prior to assessment of immunogenicity, induction of serum opsonic antibodies, and protection against GAS infection upon challenge.

MATERIALS AND METHODS

GAS peptides. The sequence of the 8830 peptide, corresponding to the type-specific amino-terminal region of the M protein of GAS strain 8830, residues 1 to 20, is DNGKAIYERARERALQELGPC. The sequence of the J8 peptide is QAEDKVKQ**SREAKKQVEKAL**KQLEDKVQ, corresponding to amino acid residues 344 to 355, inclusive of the conserved carboxy-terminal C region, of the M protein of the M1 GAS strain. The J8 peptide used is a chimeric peptide that contains 12 amino acids from the M protein C region (boldfaced in the sequence) and is flanked by yeast-derived GCN4 sequences, which was necessary in order to maintain the correct helical folding and conformational structure of the peptide (38).

Synthesis and purification of peptides. Peptides were synthesised by manual solid-phase peptide synthesis using Boc (*tert*-butoxycarbonyl) chemistry as described by Schnölzer et al. (41). Following synthesis, peptides were removed from the resin by hydrofluoric acid cleavage; the crude peptides were precipitated with ice-cold ethyl ether, filtered, and redissolved in 50% acetonitrile; and the resin was removed by filtration. The reaction mixture was lyophilized. Peptide purification was carried out on a Waters high-pressure liquid chromatography (HPLC) system (model 600 controller, 490 E UV detector, 60 F pump) using a preparative Vydac protein and peptide C_{18} column (2 by 25 cm). Separation was achieved with a solvent gradient beginning with 0% acetonitrile and increasing constantly to 90% acetonitrile for 40 min at a constant flow of 12 ml per min. Peptides 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 SIL-6b autoinjector with SCL-6B system controller) using a Vydac C_{18} column (5-μm pore size; 0.46 by 25 cm). Separation was achieved with a solvent gradient beginning with 0% acetonitrile and increasing constantly to 90% acetonitrile for 30 min at a constant flow of 1 ml per min. Peptides were detected at 214 nm.

Synthesis of the LCP-8830-J8 (LCP-GAS) formulation. Boc-L-amino acids and 4-methylbenzhydrylamine (MBHA) resin were purchased from Novabiochem (Läufelfingen, Switzerland). Racemic lipoamino acids were synthesized with Boc protection according to the procedures of Gibbons et al. (17). For synthesis of the LCP-8830-J8 formulation (also referred to as LCP-GAS) containing two copies each of the 8830 GAS peptide and the J8 GAS peptide, preactivated Boc-Gly-OH was coupled to the MBHA resin (0.67 mmol/g) in the first step. The next two cycles were carried out with Boc-lipoamino acids containing 8 carbon

atoms (C_8) {-HN-CH[$(CH_2)_5CH_3$]-OH}, which was followed sequentially by the coupling of Boc-Gly-OH, Boc-C_8-OH, and Boc-Lys(Boc)-OH. Thus, the lipophilic anchor was constructed by using three copies of racemic 2-aminooctanoic lipoamino acids. After deprotection of the lysine α and ε amino groups, a four-branch system was formed by coupling of Fmoc (9-fluorenyl methoxy carbonyl)-Lys(Boc)-OH to the free amino groups. The Boc-protecting groups were removed, and two identical 8830 peptide sequences were coupled directly onto the branched lysine core, with the appropriate protecting groups applied on the side chains of the amino acids. After synthesis of the two copies of the 8830 GAS peptide was completed, the Boc-protecting groups were removed and the free N-terminal groups were acetylated with a mixture of 2.5 mmol of acetic anhydride and 2.5 mmol of diisopropylethylamine in dimethylformamide, resulting in the (acetyl-8830 peptide)$_2$ (Fmoc)$_2$ (═Lys)$_2$═Lys-C_8-Gly-C_8-C_8-Gly-MBHA resin. After removal of the Fmoc-protecting groups by mixing the peptide-resin in 20% piperidine in DMF for 20 min, the J8 GAS peptide was assembled on each of the two new branches, with the appropriate protecting groups applied on the side chains of the amino acids. The chemical structure of the LCP-8830-J8 formulation is shown in Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resulted in bands of the expected sizes for LCP-GAS. The mass spectrum of LCP-8830-J8 was calculated as 12,373.11 Da.

Mice and immunization. All protocols were approved by the Bancroft Centre Research Ethics Committee and were carried out according to Australian National Health and Medical Research guidelines. Four- to 6-week-old female B10.BR mice (Animal Resource Centre, Perth, Western Australia, Australia) were used (n = 10 per group) in two separate experiments for subcutaneous immunization at the tail base on day 0 with 30 μg of LCP-GAS, either emulsified 1:1 with CFA (Sigma) (LCP-GAS–CFA) or given alone in a total volume of 50 μl of sterile-filtered phosphate-buffered saline (PBS) (LCP-GAS–PBS). After 3 weeks, mice received five further boosts at weekly intervals (days 21, 28, 35, 42, and 49) with 3 μg of LCP-GAS–PBS prior to collection of blood. Controls received either 30 μg of the 8830 or the J8 GAS peptide in CFA with boosts of 3 μg in PBS or CFA-PBS alone.

Collection of sera. Blood was collected 1 week after the last immunization (day 56) from each mouse by the tail artery and was allowed to clot at 37°C for 1 h, followed by removal of clots by centrifugation at 3,000 rpm for 10 min. Sera were then stored at −20°C.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed for measurement of total immunoglobulin G (IgG) antibody titers in sera, essentially as described previously (37). Serum samples were assayed by using twofold dilutions of a 1:100 dilution of serum. For IgG isotyping, horseradish peroxidase-conjugated sheep anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies (The Binding Site, Birmingham, United Kingdom) were used as the secondary antibodies. 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).

Bactericidal assay. Serum opsonic antibodies against the 8830 and M1 GAS strains were detected by a standard bactericidal assay, as described previously (37). Briefly, GAS were incubated end over end at 37°C for 3 h in the presence of nonopsonic human donor blood and either heat-inactivated (60°C for 10 min) mouse immune serum or control normal mouse serum. GAS were then plated in duplicate on 2% (vol/vol) horse blood agar plates, and colonies were counted after 24 h of incubation. The percentage of GAS opsonized was determined by counting the number of colonies growing after incubation with test immune serum and comparing this with the number of colonies growing after incubation with control serum. Opsonic activity (percent reduction in mean CFU) was calculated as (1 − CFU in the presence of immune serum)/(mean CFU in the presence of normal mouse serum) × 100.

Peptide inhibition bactericidal assay. The peptide inhibition bactericidal assay was performed essentially as described above except that sera were initially incubated for 30 min at room temperature with 100 μg of peptide prior to incubation with GAS and nonopsonic donor blood. Peptides used were the 8830 GAS peptide, the J8 GAS peptide, and a nonspecific control peptide from a *Schistosoma* sp. (EGKVSTLPLDIQIIAATMSK). The percent inhibition of opsonization was calculated as (CFU in immune sera with peptide − CFU in immune sera with no peptide)/(CFU in normal mouse sera with no peptide − CFU in immune sera with no peptide) × 100.

Western blot analysis. Mouse LCP-8830-J8 antisera were tested for cross-reactivity to four different human heart preparations (two heart and two mitral valve extracts) obtained from patients undergoing heart transplant surgery at The Prince Charles Hospital (with ethics approval from The Prince Charles Hospital Foundation), porcine heart myosin (Sigma), and porcine muscle tropomyosin (Sigma) by standard SDS-PAGE and Western blot analysis (37). The J8 GAS peptide was conjugated to diptheria toxoid and included as a positive control. Prestained kaleidoscope protein size markers were used (Bio-Rad, Hercules, Calif.). Briefly, 5 μg of each protein was electrophoresed on two commercially available precast 4 to 20% Gradigels (Gradipore); one gel was stained with Coomassie blue and destained in 40% methanol–10% acetic acid, and the second gel was blotted onto a PVDF-Plus transfer membrane (Micron Separations Inc., Westborough, Mass.) according to the manufacturer's instructions. The antiserum was used as the primary antibody at a dilution of 1:1,500, and the secondary sheep anti-mouse IgG antibody (Silenus Pty Ltd., Melbourne, Victoria, Australia) was used at a dilution of 1:1,500.

Growth of GAS and intraperitoneal challenge. GAS strain 8830 (obtained from clinical isolates in the Northern Territory of Australia and verified by M protein N-terminal sequencing) which had been serially passaged in mouse spleens to enhance virulence was used in the challenge experiments. The GAS strain was cultured overnight at 37°C in Todd-Hewitt broth (THB)–1% neopeptone. Bacteria were collected by centrifugation for 10 min at 3,000 rpm (Beckman model 9S-6R centrifuge) and washed twice with THB–1% neopeptone prior to resuspension in 25% of the original volume. Bacteria were then serially 10-fold diluted in broth and plated onto 2% blood–TH agar plates in order to estimate the inoculum size (in CFU per milliliter) after overnight incubation at 37°C. Two weeks after the last boost (day 63), mice were challenged intraperitoneally with 400 μl (1×10^5 CFU/ml in experiment 1; 2.5×10^4 CFU/ml in experiment 2) of GAS 8830. The dose of GAS had previously been shown to kill approximately 90% of naive animals. According to Institutional Ethics Committee requirements, mice that were found to be moribund were euthanized.

Statistical analysis. The Wilcoxon rank sum statistic was used for comparison of antibody titers and percent opsonization levels in the different groups. SPSS, release 10.0, was used for statistical analysis. Fisher's exact test was used to compare the proportions of surviving mice in the different experimental groups. For survival analysis (time to death), the log rank test was used to compare the differences in survival times in the different groups. Resulting P values below 0.05 were taken to indicate statistically significant differences.

RESULTS

Serum IgG antibody responses in B10.BR mice immunized with LCP-8830-J8. To assess the immunogenicity of the LCP-8830-J8 formulation, which contains an M protein amino-terminal type-specific peptide sequence (8830) in combination with a conserved non-host-cross-reactive carboxy-terminal C-region peptide sequence (J8) of the M protein (Fig. 1), serum IgG antibody responses to GAS peptides 8830 and J8 were determined for mice immunized with LCP-GAS, either with (LCP-GAS–CFA) or without (LCP-GAS–PBS) CFA, in a duplicate experiment (Fig. 2). In both experiments, in which mice were to be challenged with GAS strain 8830, the 8830 GAS peptide was coadministered in CFA as a positive control. A J8 GAS peptide-CFA-immunized control group was included in experiment 1. Three weeks after the primary immunization, 8830 and J8 GAS peptide-specific antibodies were detected in all mice immunized with LCP-GAS–CFA, giving final average titers of antibodies to the 8830 and J8 GAS peptides, after five boosts, of 2.18×10^6 and 2.55×10^6, respectively (experiment 1; Fig. 2A and B). No antibodies specific to the 8830 and J8 GAS peptides were detected at 3 weeks postimmunization in mice immunized with LCP-GAS–PBS. However, after one boost of immunogen, 8 of the 10 mice had 8830 GAS peptide-specific antibodies and 3 mice had J8 GAS peptide-specific antibodies. After the third boost of immunogen, antibodies to the 8830 GAS peptide were detected in all mice and eight of the mice demonstrated a response to the J8 peptide. After the final boost (boost 5), the average 8830 and J8 GAS peptide-specific antibody titers in mice immunized with LCP-GAS–PBS were 1.04×10^5 and 6.91×10^3 (experiment 1), respectively.

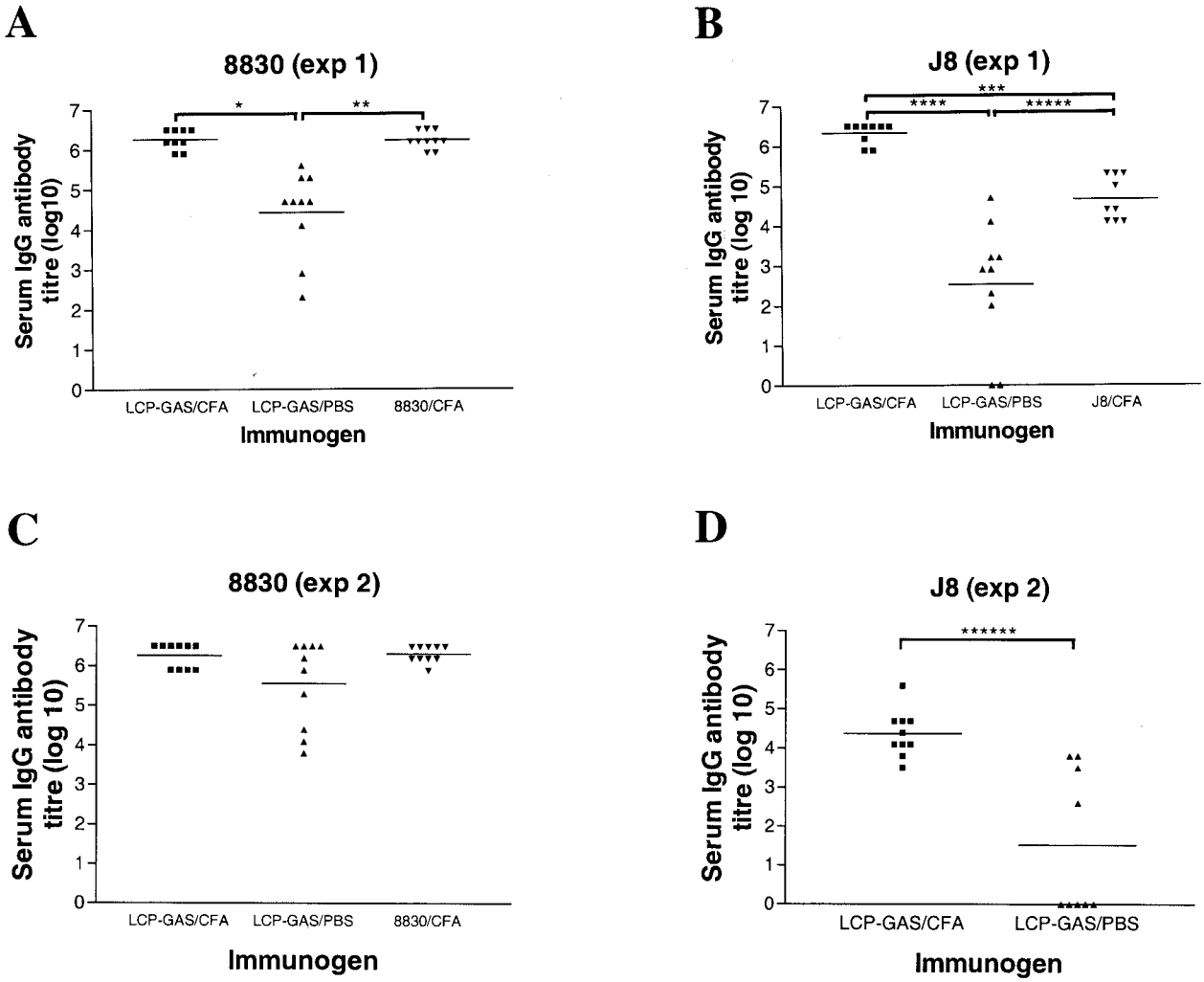

FIG. 2. Serum IgG antibody responses in B10.BR mice immunized parenterally with the LCP-8830-J8 (LCP-GAS) formulation in the presence (LCP-GAS–CFA) or absence (LCP-GAS–PBS) of CFA, for experiment 1 (A and B) and experiment 2 (C and D). Antibody titers to the 8830 and J8 GAS peptides for individual mice are shown, with the average titer (geometric mean) indicated by a bar. Antibody titers to the 8830 and J8 peptides are shown for individual control mice that were immunized with the 8830 peptide in CFA or the J8 peptide in CFA, respectively. By use of the Wilcoxon rank sum statistic, mean antibody titers were significantly different in the different immunized groups: *, $P < 0.0001$; **, $P < 0.0001$; ***, $P < 0.0001$; ****, $P < 0.0001$; *****, $P = 0.002$; ******, $P = 0.001$.

In the second experiment (Fig. 2C and D), in which mice also received a primary immunization and five boosts each of the same immunogen, the final average 8830 and J8 GAS peptide-specific serum IgG antibody titers were 2.29×10^6 and 6.34×10^4, respectively, for mice immunized with LCP-GAS–CFA. For mice immunized with LCP-GAS–PBS, the average titers of antibody to the 8830 and J8 GAS peptides were 1.39×10^6 and 1.80×10^3, respectively. For 8830 GAS peptide-CFA-immunized control mice, average 8830 peptide-specific serum IgG antibody titers were 1.97×10^6 (experiment 1) and 2.38×10^6 (experiment 2). While immunization with LCP-GAS–CFA produced antibody responses to the 8830 peptide similar to those of the control group given monomeric 8830 peptide alone in CFA, the response to the J8 peptide was substantially higher than that of mice administered J8 peptide alone in CFA ($P < 0.0001$) (Fig. 2B). We observed that the immunogenicity of LCP-GAS for the J8 peptide was lower in experiment 2 than in experiment 1 (titers, 6.34×10^4 versus 2.55×10^6 in CFA; 1.80×10^3 versus 6.91×10^3 in PBS) (Fig. 2B and D). The possibility of antigen degradation during storage of the vaccine was not an issue, because the immunogenicity of LCP-GAS after 8 months' storage yielded final average J8 GAS peptide-specific serum IgG antibody titers of 2.46×10^6 and 6.68×10^4 in LCP-GAS–CFA- and LCP-GAS–PBS-immunized mice, respectively (data not shown); these were comparable to the titers obtained in experiment 1 after storage for 1 month.

Overall, the antibody responses to the 8830 GAS peptide were not significantly different following immunization with LCP-GAS–CFA or 8830 GAS peptide-CFA. Antibody responses to the J8 GAS peptide, however, were significantly elevated in LCP-GAS–CFA-immunized mice relative to those in the J8 GAS peptide-CFA-immunized group. In mice immunized with LCP-GAS–PBS, the antibody responses to both the 8830 and J8 GAS peptides were generally lower than those in the LCP-GAS–CFA and peptide-CFA groups, although some mice exhibited particularly high-titer antibody responses to the

TABLE 1. Serum 8830-specific IgG isotype antibody responses in B10.BR mice immunized parenterally with LCP-8830-J8–CFA, LCP-8830-J8, or 8830-CFA

Immunogen	Mouse	Titer[a] of:							
		IgG1		IgG2a		IgG2b		IgG3	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
LCP-8830-J8–CFA	M1	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	0
	M2	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	1,600
	M3	>12,800	>12,800	>12,800	12,800	>12,800	>12,800	3,200	200
	M4	>12,800	>12,800	>12,800	6,400	>12,800	>12,800	3,200	1,600
	M5	>12,800	>12,800	>12,800	6,400	>12,800	>12,800	3,200	200
	M6	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	200
	M7[b]		>12,800		>12,800		>12,800		200
	M8	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	1,600
	M9	>12,800	>12,800	>12,800	3,200	>12,800	>12,800	3,200	200
	M10	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	12,800
LCP-8830-J8	M1	>12,800	>12,800	3,200	0	3,200	12,800	400	0
	M2	>12,800	>12,800	3,200	6,400	200	12,800	100	0
	M3	100	>12,800	0	0	0	3,200	0	0
	M4	>12,800	>12,800	3,200	0	3,200	400	400	0
	M5	>12,800	>12,800	3,200	1,600	>12,800	12,800	800	0
	M6[b]	>12,800		3,200		6,400		400	
	M7	>12,800	>12,800	100	200	200	200	0	0
	M8	>12,800	>12,800	800	0	3,200	0	200	0
	M9	>12,800	>12,800	800	0	200	400	100	0
	M10	200	>12,800	0	0	0	0	0	0
8830-CFA	M1	>12,800	>12,800	>12,800	12,800	>12,800	>12,800	1,600	100
	M2	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	1,600	100
	M3	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	100
	M4	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	100
	M5	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	200
	M6	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	200
	M7	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	100
	M8	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	200
	M9	6,400	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	100
	M10	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	3,200	1,600

[a] IgG1, IgG2a, IgG2b, and IgG3 titers are shown for individual mice for experiments 1 and 2.
[b] Mouse 7 (LCP-GAS–CFA group, experiment 1) and mouse 6 (LCP-GAS—PBS group, experiment 2) died prior to analysis.

8830 peptide (Fig. 2C). It should be emphasized that CFA is considered the "gold standard" adjuvant in that it induces exceptionally high immune responses.

Serum IgG isotype antibody response in B10.BR mice immunized with LCP-8830-J8. To investigate further the nature of the peptide-specific serum IgG antibody response induced following parenteral delivery of LCP-GAS, we assessed the IgG subclass types that were elicited by the 8830 and J8 GAS peptides (Tables 1 and 2). In LCP-GAS–CFA-immunized mice, high titers of 8830 and J8 peptide-specific IgG1, IgG2a, and IgG2b antibodies were detected in the majority of mice, with lower titers detected for IgG3. Similarly, control mice immunized with monomeric peptide (8830) in CFA, or J8 in CFA, also showed high titers of IgG1, IgG2a, and IgG2b antibodies, with lower IgG3 antibody titers. For mice immunized with LCP-GAS–PBS, there was a preponderance of 8830 peptide-specific IgG1 antibodies elicited, whereas titers of 8830 peptide-specific IgG2a, IgG2b, and IgG3 antibodies were lower than those for mice immunized with LCP-GAS–CFA or 8830 peptide-CFA. The lower antibody titers most likely reflect the lower 8830 peptide-specific serum IgG antibody titers detected for this group relative to those for mice immunized with LCP-GAS–CFA (1.04×10^5 versus 2.18×10^6 in experiment 1; 1.39×10^6 versus 2.29×10^6 in experiment 2). The serum IgG antibody response to the J8 peptide in LCP-GAS–PBS-immunized mice showed a preponderance of IgG1 in experiment 2. However, very little J8 peptide-specific antibody of any IgG isotype was detected in experiment 1. This is most likely due to the lower titers of J8 peptide-specific IgG antibodies detected in the sera of LCP-GAS–PBS-immunized mice relative to those in mice immunized with LCP-GAS–CFA (averages, 6.91×10^3 versus 2.55×10^6 in experiment 1; 1.80×10^3 versus 6.34×10^4 in experiment 2).

Overall, the IgG isotype responses to the 8830 and J8 GAS peptides were similar following immunization with LCP-GAS–CFA or peptide-CFA, with the general trend of higher peptide-specific IgG1, IgG2a, and IgG2b antibodies and lower peptide-specific IgG3 antibodies. In mice immunized with LCP-GAS–PBS, antibody responses to the 8830 peptide were highest for IgG1, with IgG2b, IgG2a, and IgG3 following in that order, whereas a predominantly IgG1 response to J8 peptide was observed, with few mice giving an IgG2a or IgG2b response.

Induction of opsonic antibodies in B10.BR mice immunized with LCP-8830-J8. Using an indirect bactericidal assay, we assessed the opsonic activity toward GAS strain 8830 of serum

TABLE 2. Serum J8-specific IgG isotype antibody responses in B10.BR mice immunized parenterally with LCP-8830-J8–CFA, LCP-8830-J8, or J8-CFA

Immunogen	Mouse	Titer[a] of:							
		IgG1		IgG2a		IgG2b		IgG3	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
LCP-8830-J8–CFA	M1	>12,800	>12,800	3,200	3,200	>12,800	>12,800	3,200	0
	M2	>12,800	>12,800	3,200	800	>12,800	3,200	1,600	0
	M3	>12,800	>12,800	3,200	6,400	>12,800	>12,800	1,600	0
	M4	>12,800	>12,800	6,400	3,200	>12,800	>12,800	1,600	0
	M5	>12,800	>12,800	3,200	>12,800	>12,800	3,200	1,600	0
	M6	>12,800	>12,800	6,400	>12,800	>12,800	>12,800	3,200	100
	M7[b]		>12,800		3,200		>12,800		0
	M8	>12,800	>12,800	3,200	0	>12,800	3,200	1,600	0
	M9	>12,800	>12,800	6,400	>12,800	>12,800	>12,800	1,600	100
	M10	>12,800	>12,800	3,200	>12,800	>12,800	>12,800	1,600	100
LCP-8830-J8	M1	3,200	>12,800	3,200	0	400	0	0	0
	M2	400	>12,800	100	0	0	0	0	0
	M3	0	3,200	0	0	0	0	0	0
	M4	0	>12,800	100	100	0	>12,800	0	0
	M5	100	>12,800	100	0	100	100	0	0
	M6[b]	100		0		0		0	
	M7	0	800	0	0	0	0	0	0
	M8	3,200	3,200	800	0	400	0	0	0
	M9	0	>12,800	0	0	0	>12,800	0	0
	M10	0	3,200	0	0	0	100	0	0
J8–CFA	M1	>12,800		3,200		6,400		400	
	M2	>12,800		>12,800		6,400		800	
	M3	>12,800		320		1,600		400	
	M4	1,600		3,200		3,200		400	
	M5	>12,800		3,200		1,600		400	
	M6	1,600		>12,800		3,200		400	
	M7	>12,800		>12,800		6,400		800	
	M8	>12,800		>12,800		3,200		800	
	M9	>12,800		3,200		1,600		400	
	M10[a]								

[a] IgG1, IgG2a, IgG2b, and IgG3 titers are shown for individual mice for experiments 1 and 2.
[b] Mouse 7 (LCP-GAS–CFA group, experiment 1), mouse 6 (LCP-GAS–PBS group, experiment 2), and mouse 10 (J8-CFA group) died prior to analysis.

IgG antibodies elicited after parenteral delivery of LCP-8830-J8 (Fig. 3A and C; Table 3). The average percent opsonization of GAS 8830 by LCP-GAS–CFA antisera was 96% (82% for LCP-GAS–PBS) for experiment 1 and 93% (68% for LCP-GAS–PBS) for experiment 2. There was complete opsonization of GAS by control 8830 peptide-CFA antisera in experiment 1 and 93% opsonization by control antisera in experiment 2. We also assessed the potential of antisera generated in experiment 1 for opsonizing another GAS strain, M1 (Fig. 3B and Table 3). Unlike the relatively low opsonization of GAS by antibodies induced to the C-region J8 peptide epitope (23%) following immunization with J8 peptide-CFA, positive-control antisera against pepM1 (the amino-terminal half of M1 protein) completely opsonized GAS M1. The average percent opsonization for LCP-GAS–CFA antisera against GAS M1 was 67% (48% for LCP-GAS–PBS). There was a low level of opsonization (10%) for 8830 peptide-CFA antisera against the heterologous GAS M1 strain, demonstrating a lack of cross-reactivity between type-specific M protein determinants. These findings indicate that opsonization of GAS M1 by LCP-GAS antisera was mediated partly by antibodies directed against the J8 conserved C-region peptide epitope on GAS.

To confirm the specificity of the serum opsonic antibodies induced in LCP-GAS-immunized mice, a peptide inhibition bactericidal assay was performed which involved preincubation of immune sera with either the 8830 peptide, the J8 peptide, or a nonspecific control peptide derived from *Schistosoma* prior to assessment of opsonization. The percent inhibition of opsonization was then determined by comparing the percentage of opsonization in the presence of the peptide to that observed in the absence of the peptide. Opsonization of GAS 8830 by pooled LCP-GAS–CFA or LCP-GAS–PBS antisera in the absence of peptide was 100 or 95%, respectively. Preincubation of LCP-GAS–CFA antisera with the 8830 peptide, however, led to almost complete inhibition (89%) of opsonization. LCP-GAS antisera preincubated with the 8830 peptide showed 78% inhibition of opsonization. The nonspecific peptide and the J8 peptide did not inhibit the opsonization of GAS 8830 by LCP-GAS–CFA and LCP-GAS antisera.

Together, these data indicate that the LCP-8830-J8 formulation induced serum opsonic antibodies specifically directed against the M protein type-specific peptide epitope on GAS strain 8830.

Lack of heart-cross-reactive antibodies. The GAS M protein (12), by virtue of its homology with various structural heart antigens, may mediate autoimmune responses and play a role in the pathogenesis of RHD. It was therefore necessary to assess whether heart-cross-reactive antibodies were elicited in

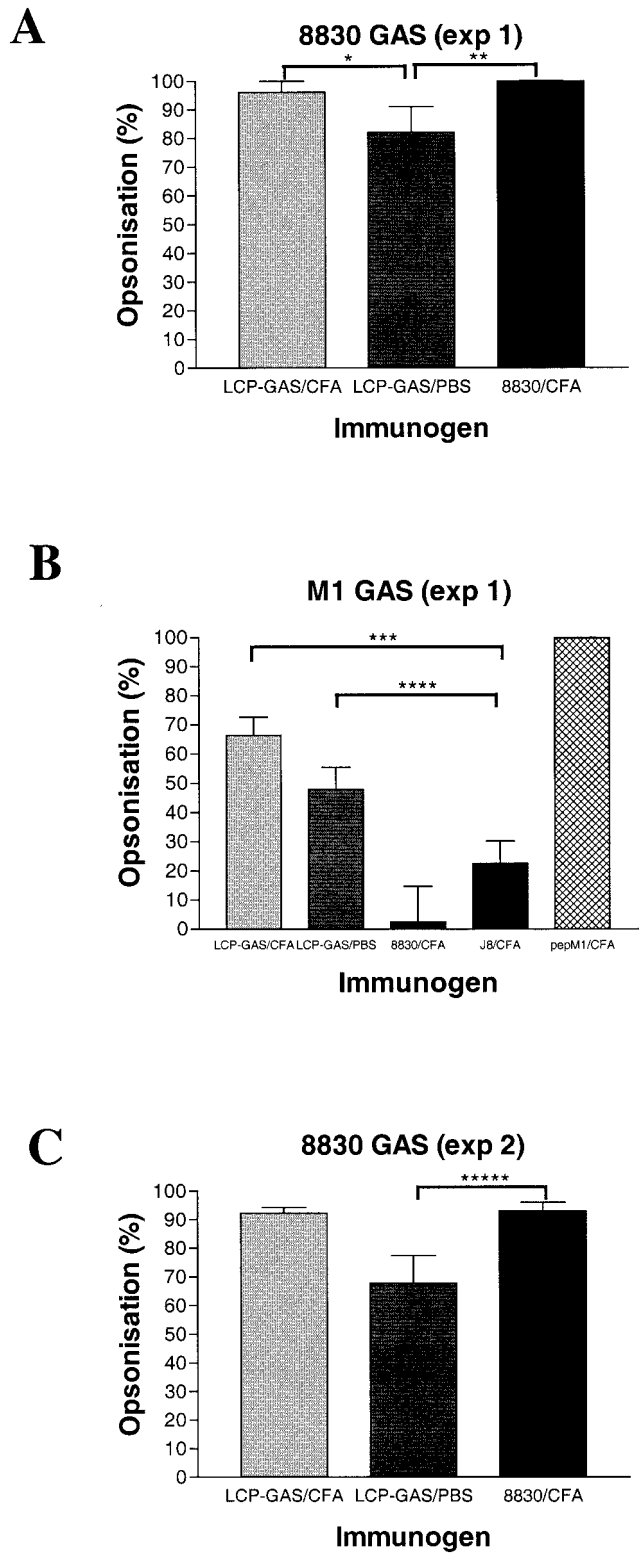

FIG. 3. Opsonization of GAS strains 8830 and M1 by LCP-GAS–CFA, LCP-GAS–PBS, and control (8830 peptide-CFA or J8 peptide-CFA) B10.BR mouse antisera in experiments 1 (A and B) and 2 (C). The average percent opsonization (measured as the percent reduction in CFU of bacteria) for each individual group is shown. Error bars, standard errors of the means. Opsonization was determined by an indirect bactericidal assay which compares the growth of bacteria (expressed in CFU) following incubation in the presence of immune sera to that with control normal mouse sera obtained from mice immunized with adjuvant in PBS alone. By use of the Wilcoxon rank sum statistic, mean opsonization levels for the different immunized groups were significantly different: *, $P = 0.036$; **, $P = 0.004$; ***, $P = 0.002$; ****, $P = 0.037$; *****, $P = 0.041$.

response to immunization with the LCP-8830-J8 formulation. Sera from mice immunized parenterally with LCP-8830-J8 were tested for the presence of cross-reactive antibodies to human heart and mitral valve extracts by use of standard SDS-PAGE and Western blot analysis. J8 conjugated to diptheria toxoid was included as a positive control and was recognized strongly by LCP-8830-J8 antisera. No reactivity, however, was observed to any of the heart tissue proteins (data not shown).

Survival of mice immunized with LCP-8830-J8 and challenged with GAS. To determine whether immunization of mice with LCP-GAS, which induced production of serum opsonic antibodies, also induced protective immunity to GAS, mice were challenged in vivo with GAS strain 8830 (10^5 and 2.5×10^4 CFU/ml for experiments 1 and 2, respectively) and the percent survival was determined up to 10 days postchallenge. Compared to the CFA-PBS control group, in which 11% of the mice (1 of 9) survived, the percent survival of mice immunized with LCP-GAS–CFA was 90% (8 of 9) ($P = 0.003$) (Fig. 4). There was complete protection of mice (10 of 10) immunized with LCP-GAS–PBS ($P < 0.0001$ versus the CFA-PBS group). Survival was 90% (9 of 10) in 8830 peptide-CFA-immunized mice ($P = 0.001$ for comparison to CFA-PBS control mice). Analysis of the survival times of these groups compared to those for CFA-PBS control mice also showed significantly increased survival for mice immunized with LCP-GAS–CFA ($P = 0.0013$), LCP-GAS–PBS ($P = 0.0001$), or 8830 peptide-CFA ($P = 0.0008$). In experiment 2, compared to the CFA-PBS control group, in which 50% (5 of 10) of mice survived, the percent survival of mice immunized with LCP-GAS–CFA was 100% (10 of 10) ($P = 0.033$) (Fig. 4). There was complete protection of mice (9 of 9) immunized with LCP-GAS–PBS ($P = 0.033$). Survival was 100% (10 of 10) in 8830 peptide-CFA-immunized mice ($P = 0.033$ for comparison to the CFA-PBS group). Analysis of the survival times for these groups compared to those for CFA-PBS control mice also showed significantly increased survival for mice immunized with LCP-GAS–CFA ($P = 0.011$), LCP-GAS–PBS ($P = 0.017$), or 8830 peptide-CFA ($P = 0.011$). In experiment 2, two groups of mice were also immunized with an LCP formulation containing four copies of a control peptide from *Schistosoma* (referred to as LCP-SP) in the presence or absence of CFA. Survival in these groups was 55% (5 of 9) and 44% (4 of 9) for LCP-SP–CFA- and LCP-SP–PBS-immunized mice, respectively, and was not significantly different from that for control CFA-PBS-immunized mice. Analysis of the survival times for these groups compared to those for CFA-PBS control mice also showed no significant differences.

DISCUSSION

Development of synthetic peptide-based immunogens is emerging as a possible approach for human vaccination in the future, as indicated particularly by recent reports of experi-

TABLE 3. Opsonization of GAS by LCP-GAS and control antisera[a]

GAS strain	No. of bacteria in GAS inoculum (CFU)	No. of bacteria in normal mouse serum (mean CFU)	Immunogen	No. of bacteria in immune serum (mean CFU)	% Average population reduction
8830 (expt 1)	62	84,004	LCP-GAS–CFA	3,251	96
			LCP-GAS–PBS	15,004	82
			8830–CFA	132	100
M1	107	35,724	LCP-GAS–CFA	11,937	67
			LCP-GAS–PBS	18,590	48
			J8-CFA	27,675	23
			pepM1-CFA	2	100
			8830-CFA	34,890	2
8830 (expt 2)	76	450,750	LCP-GAS–CFA	31,529	93
			LCP-GAS–PBS	132,792	68
			8830–CFA	31,024	93

[a] By use of an indirect bactericidal assay and GAS strains 8830 and M1, mean bacterial-population counts (in CFU) from individual serum samples plated out in duplicate were determined following incubation of GAS of a known inoculum size in the presence of immune sera (LCP-GAS–CFA, LCP-GAS–PBS, 8830-CFA, J8-CFA, or pepM1-CFA) and of control normal mouse sera obtained from mice immunized with CFA in PBS alone. Average percentages of bacterial-population reduction were determined by comparing the mean CFU in immune sera to those in control sera.

mental models demonstrating the potential of synthetic polymeric (22), linear and nonlinear (branched) B-cell-T-cell (14, 16), and synthetic lipid-containing peptide immunogens (1, 11, 24, 27–31, 39, 40, 45–48; reviewed in reference 2) as alternatives to conventional vaccine delivery. In this study we report on the synthesis, immunogenicity, and protective potential of a novel LCP-GAS vaccine candidate incorporating two different M protein protective peptide epitopes. The α-helical coiled-coil M protein consists of a highly variable amino-terminal region, which defines the GAS serotype, and a highly conserved C region (12) and remains the major candidate in vaccination against GAS infection. Development of a broad-strain-coverage, M protein-based GAS vaccine has proven difficult due to the sequence variability of different M proteins (more than 100 serotypes are known) and the potential for induction of autoimmunity due to antigenic molecular mimicry with self antigen (15). Our data have shown that immunization of mice with an LCP-GAS formulation (LCP-8830-J8) containing an M protein type-specific amino-terminal peptide sequence (8830) and a non-host-cross-reactive carboxy-terminal conserved C-region peptide sequence (J8) elicited high-titer serum IgG antibodies to both the 8830 and J8 GAS peptides in the presence and absence of CFA and that this formulation did not induce antibodies to cross-react with human heart tissue proteins. The peptide-specific serum IgG antibody subtypes induced were predominantly IgG1, IgG2a, and IgG2b for LCP-GAS–CFA-immunized mice and predominantly IgG1 for mice immunized with LCP-GAS–PBS, thus favoring Th2 responses. It remains to be determined whether the LCP system could be combined with other, more Th1 adjuvants to further increase the potential usefulness of this delivery system.

Using an indirect bactericidal assay, we have further demonstrated induction of opsonic antibodies to two different GAS strains, 8830 and M1, following immunization with LCP-8830-J8, again in the presence or absence of CFA. The opsonization of GAS strains 8830 and M1 by LCP-GAS–CFA and LCP-GAS–PBS antisera indicates that serum opsonic antibodies to the type-specific amino-terminal and conserved C-regions of the M protein were induced in mice following immunization with LCP-GAS. Moreover, induction of opsonic antibodies was not dependent on the presence of conventional adjuvant, as demonstrated by opsonization of GAS strains 8830 and M1 by antisera from LCP-GAS–PBS-immunized mice, supporting the efficacy of the LCP system as a self-adjuvanting vaccine delivery modality. The ability of the 8830 GAS peptide to inhibit opsonization of GAS strain 8830 by LCP-GAS–CFA and LCP-GAS–PBS antisera indicates that the LCP-GAS formulation induced serum opsonic antibodies specifically directed against the M protein type-specific peptide epitope on GAS and may potentially be important in protective immunity to GAS infection. The inability of the J8 peptide to inhibit the opsonization of GAS 8830 indicates that opsonic antibodies generated to the C region of the M protein are not necessary for the opsonization of GAS 8830 in the presence of type-specific opsonic antibodies. We have recently shown, however, that an LCP compound comprised solely of J8 induced serum J8-specific heterologous opsonic antibodies (31) which may be involved in mediating broadly protective immune responses.

Upon challenge of mice with GAS strain 8830 in two separate experiments, animals were completely protected from GAS infection. These results indicate that immunization with LCP-GAS induced type-specific opsonic antibodies that are important for protective immunity to GAS, and they support our earlier findings and those of others demonstrating the efficacy of the LCP system as a vaccine delivery approach for enhancing the immunogenicity of various synthetic peptide immunogens (31, 46, 48) including viral and bacterially derived antigens. It is noteworthy that while serum IgG responses and opsonic antibodies induced in response to LCP-GAS–CFA were greater than those with LCP-GAS given in the absence of adjuvant, equivalent levels of survival were attained. This may indicate that sufficient levels of antibody were elicited for protection or that additional factors may contribute to the protective mechanisms involved.

Previous strategies to develop a broad-strain-coverage GAS vaccine have included the design of recombinant multivalent M protein vaccine constructs containing epitopes from the amino-terminal type-specific regions of several different M protein serotypes (6, 10, 21) and the identification of vaccine candidates based on the conserved C region of the M protein (6, 8, 31, 32). Peptide J8 has been identified as the minimal murine protective B-cell epitope in the M protein C region

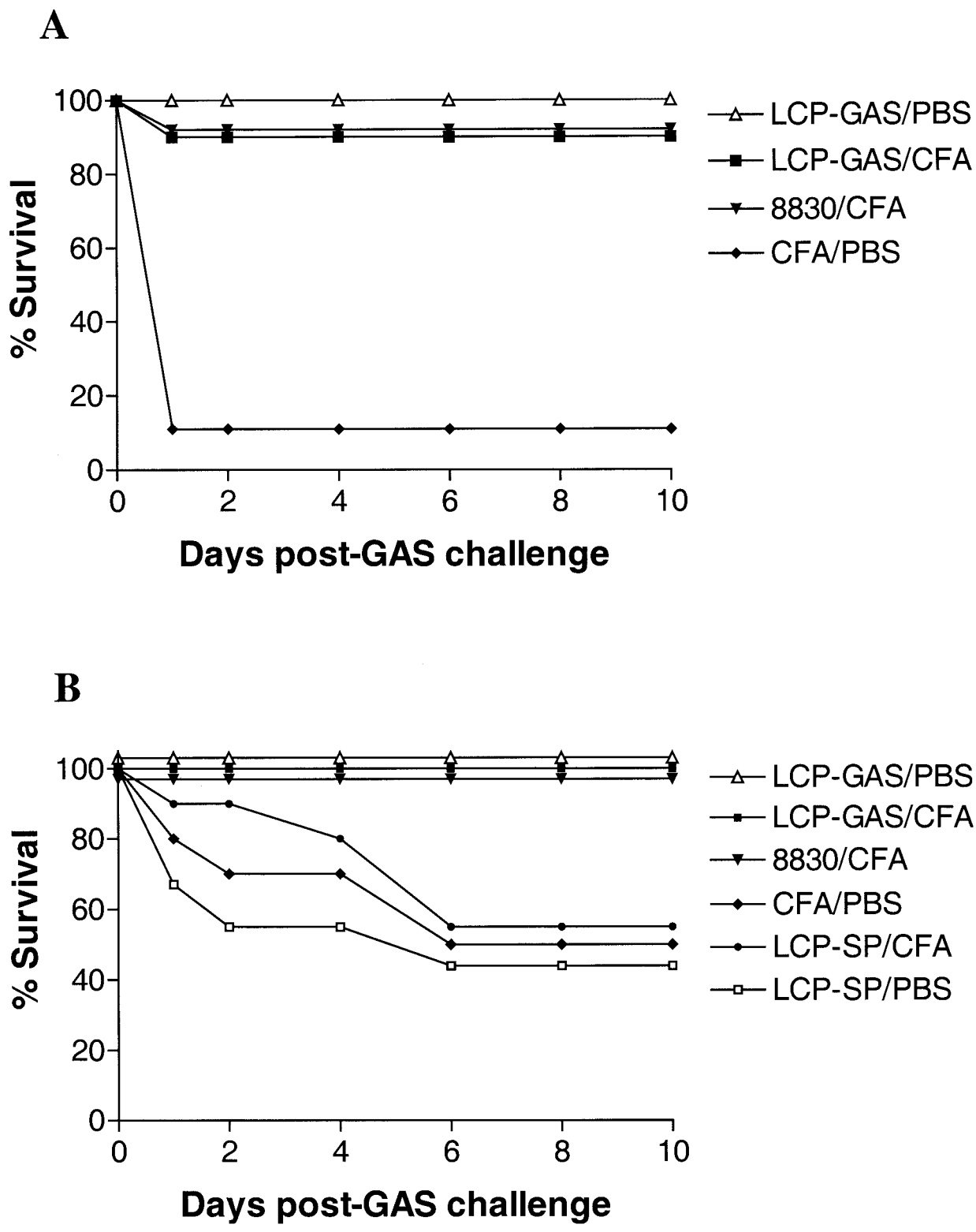

FIG. 4. Survival curves for mice immunized with LCP-GAS–CFA, LCP-GAS–PBS, and control 8830 peptide-CFA following parenteral GAS challenge in two separate experiments. By use of Fisher's exact test to compare the proportions of surviving mice, significantly greater survival was observed for mice immunized with LCP-GAS–CFA or LCP-GAS–PBS than for control CFA-PBS-immunized mice ($P = 0.003$ and $P < 0.0001$, respectively, for experiment 1; $P = 0.033$ for experiment 2).

(19). It has recently been reported that the J8 peptide significantly protected mice against GAS infection following mucosal immunization and challenge (32). The vaccine required conjugation to diphtheria toxoid and was delivered in the presence of the mucosal adjuvant, cholera toxin B subunit. Other approaches which have been used for delivery of recombinant M proteins have involved their expression on commensal bacteria such as attenuated *Salmonella enterica* serovar Typhimurium (34) or *Streptococcus gordonii* (35) or on live viral vectors such as vaccinia virus (13). There are obvious safety concerns, however, associated with using pathogenic organisms as live vaccine vector delivery systems.

Dale and colleagues have developed recombinant 8- and 26-valent M protein vaccine constructs containing epitopes from the amino-terminal regions of different M protein serotypes (10, 21). In each case, the vaccines were shown to be immunogenic in rabbits. Furthermore, the antisera produced were opsonic against the GAS strains represented in the recombinant constructs, and there was no cross-reactivity with human tissues, indicating the potential use of these vaccines in inducing safe and protective immune responses. Theoretically, however, these vaccines would provide protection only against the GAS strains represented in the constructs. The design of a multiepitope GAS vaccine construct, referred to as "heteropolymer," targeted specifically to GAS strains commonly isolated from patients in the Australian aboriginal population of the Northern Territory, has recently been reported (6). The construct is essentially a random polymer and contains multiple M protein amino-terminal peptides and a conserved C-region peptide. Mice were completely protected following parenteral immunization with heteropolymer in CFA and challenge with two different GAS strains (6).

Although the autoimmunity consequences of these vaccines have potentially been eliminated, together these strategies are fraught with problems of strain variability, quality control (in the case of the heteropolymer, with its lack of defined order of peptides), and adjuvant toxicity that need to be overcome before progression to clinical trials.

This report indicates the potential of LCP technology as a safe and effective alternative approach to the delivery of new-generation synthetic peptide vaccines containing different protective epitopes of either the same or different target antigens. For example, the recently identified protective epitope of SfbI, an adhesin important in the colonization of human epithelial cells and in imparting protective immunity to GAS (42), could be combined with an M protein protective epitope in the design of a multivalent LCP-based GAS vaccine. Due to the self-adjuvanting properties of the LCP system, LCP-based vaccines may also have the potential for mucosal delivery of nasal vaccines against many human mucosal pathogens. Moreover, due to the defined order of unique sequences on the lipid core within the vaccines, quality control of the products is assured.

Together our findings indicate the potential of the LCP system in the delivery of self-adjuvanting multi-antigen component synthetic peptide vaccines, with a view to the development of mucosa-based vaccines for human vaccination.

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