CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Gemini lipopeptides as vaccine adjuvants: a new role for these versatile carriers

Clin Exp Vaccine Res 2020;9:159-163
https://doi.org/10.7774/cevr.2020.9.2.159
pISSN 2287-3651 • eISSN 2287-366X

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Received: December 4, 2019
Revised: February 4, 2020
Accepted: February 11, 2020

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No potential conflict of interest relevant to this article was reported.

This work was supported by grants from Universidad Nacional del LItoral (CAI+D 50120150100059LI, and Seed Capital 2018), and from the National University Policies Secretary (SPU).

The design of subunit vaccines requires new adjuvant systems. We designed and synthesized new lipopeptides (cysteine-based) of low molecular weight with different hydrophobic chains that dimerize becoming *gemini* lipopeptides. They were characterized and their adjuvant capacity was tested in mice by the inoculation of a protein antigen formulated with the lipopeptides, with and without the addition of CpG-oligodeoxynucleotides. Formulations were able to induce an immune response and produced no adverse effects. An adjuvant ability is described for the first time for this type of molecules.

Keywords: Vaccines, DNA, Adjuvants, Immunostimulants, *gemini* lipopeptides

Vaccines are one of the most significant interventions of medicine, and showed important progress from empirical formulations to rational designs, along the time. Nevertheless, adjuvant development did not experience similar progress [1].

Oligonucleotides containing non-methylated CpG sequences (CpG-ODN) are immunostimulant molecules that are added in vaccine formulations as adjuvants, mostly as phosphorothioate modified ODN (PS-CpG-ODN). These types of bonds give resistance to nucleases leading to a longer *in vivo* persistence than natural phosphodiester ODN (PO-CpG-ODN). However, the cost of production of PS-CpG-ODN is higher than PO-CpG-ODN, and it has been reported that the long-term use of modified ODN may be associated with adverse side effects in mice, and primates [2]. Those are important factors to consider when developing vaccine systems. Nevertheless, PO-CpG-ODN can be used as adjuvant if complexed with an adequate vehicle, as liposomes [3,4].

Gemini compounds are amphipathic molecules formed by a hydrophobic moiety containing two hydrocarbon chains of variable length, and two hydrophilic portions that can be peptides, among other low molecular weight groups [5].

We developed a series of monomeric lipopeptides that function as DNA and protein carriers, called [AGn-Cm]2, where AGn is the peptidic polar head group and Cm is the non-polar region, attached to the peptide N-terminus [5]. A cysteine in the peptide can form a disulphide bridge with another monomer and generates the *gemini* lipopeptide by dimerization at physiological pH.

Based on their ability to form complexes with negatively charged DNA, some of them are transfection reagents with high efficiency [5,6]. However, the use of these molecules in adjuvant formulations has not been described.

In this study, we developed formulations based on cationic *gemini* lipopeptides and

PO-CpG-ODN and we assessed their adjuvant ability to induce antibody production against a model antigen.

Three monomeric lipopeptides were synthesized: AG2-C16, AG2-C18, and AG3-C18. The polar region is a pentapeptide containing tryptophan (W), ornithine (O), and cysteine (C) (Table 1), and the N-terminal end was hydrophobized with palmitic or oleic acid (C16 or unsaturated C18, respectively).

The peptide moiety was synthesized and hydrophobized applying the strategy of 9-fluorenyl-methoxycarbonyl [7] using Rink amide 4-methylbenzydrylamine (Sigma Aldrich, St. Louis, MO, USA) as the solid support. Then, the monomeric lipopeptides were detached and the side chain protectors of the amino acids were eliminated by trifluoroacetic acid, water, ethanedithiol, and triisopropylsilane (94.5:2.5:2.5:0.5). The lipopeptides were dissolved in H_2O, lyophilized, and kept at -20°C under a nitrogen atmosphere until used.

The molecular weight (MW) was determined by electrospray ionization mass spectrometry using UPLC-MS SQD2, and the amino acid sequence was confirmed by automatic Edman degradation performed on a Shimadzu 120 PPSQ-23-A sequencer. The purity of the lipopeptides was analysed by high-performance liquid chromatography in reverse phase. Critical aggregation concentration (CAC) was assessed using a duNouy Tensiometer (CSC Scientific Co. Inc., Fairfax, VA, USA) [6].

Lipopeptides were used to formulate the antigen (bovine serum albumin, BSA) with or without an immunostimulant CpG-ODN, with the sequence 5'-tccatgacgttcctgacgtt-3' linked by phosphodiester bonds (Invitrogen, Waltham, MA, USA) [2,8].

Different formulations were prepared with lipopeptides (40 or 400 μM), CpG-ODN (7.5 μM), and BSA (100 μg/mL) in phosphate-buffered saline (0.1 M, pH 7.4) or sodium acetate buffer (0.05 M, pH 4.3), and were subjected to electrophoresis on a 2% agarose gel in the presence of GelRed (Biotium, Fremont, CA, USA). The formulations were treated with the RQ1 RNase-Free DNase (Promega, Madison, WI, USA), to assess the DNA protection ability of lipopeptides.

Six- to 8-week-old Balb/c female mice were inoculated subcutaneously, with three doses, every 3 weeks, of 10 μg of BSA (Sigma Aldrich) with each of the lipopeptides under evaluation, with and without CpG-ODN (7.5 μM), in a final volume of 100 μL. Control groups received lipopeptides with CpG-ODN, BSA, and BSA with CpG-ODN. Before each inoculation and 10 days after the last dose, a blood sample was obtained from the coccygeal vein, and the serum was stored at -20°C until used. All procedures were carried out according to the recommendations of the Guide for the Care and Use of Laboratory Animals [9] and approved by the institutional Advisory Committee on Research Ethics and Security (no., 03/16).

The production of specific antibodies was evaluated by the enzyme-linked immunosorbent assay. The 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany) were incubated with 1 μg/well of BSA, non-specific binding sites were blocked with 5% skimmed milk, and the sera were incubated at a 1/100 dilution. Bound antibodies were detected by HRP-conjugated anti-mouse immunoglobulin G (IgG) (Jackson, Baltimore, MD, USA), anti-mouse IgG1 (Santa Cruz Biotechnology Inc., La Jolla, CA, USA) or anti-mouse IgG2a (Santa Cruz Biotechnology Inc.) antibodies, followed by H_2O_2/tetramethylbenzidine (Zymed, San Francisco, CA, USA). After stopping the colour reaction with H_2SO_4 2 N, the absorbance was read at 450 nm and the results were expressed in optical density units.

Kruskal-Wallis or Mann-Whitney test were applied as appropriate, using GraphPad Prism ver. 5.00 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Differences

Table 1. Sequences and characterization of the designed lipopeptides

Monomeric lipopeptide	Sequence	Molecular weight (Da)		Net charge[a]	CAC (μM)[b]
		Calculated	ESI-MS		
AG2-C_{16}	CH3-(CH2)14-CO-WWCOO-CONH2	957.3	960.5	+2	64.2
AG2-C_{18}	CH3-(CH2)16-CO-WWCOO-CONH2	983.4	986.6	+2	21.6
AG3-C_{18}	CH3-(CH2)16-CO-WWCOOO-CONH2	1,096.5	1,099.7	+3	48

ESI-MS: molecular weight of the monomeric lipopeptides determined by ESI-MS.
ESI-MS, electrospray ionization mass spectrometry; CAC, critical aggregation concentration.
[a]Calculated at pH 7.4.
[b]Determined in buffer 15 mM HEPES pH 7.4, 10 mM DTT; measurements were made in triplicate and at room temperature (25°C). The CAC value for each compound was estimated from the breaking point of the curve of surface tension as a function of concentration.

 https://doi.org/10.7774/cevr.2020.9.2.159

were significant when $p<0.05$.

The monomeric lipopeptides were synthesized and obtained with high purity (>95%), the mass-average MW obtained by mass spectrometry was close to the calculated value. Amphiphiles have a net positive charge between +2 and +3 at pH 7.4, due to the presence of ornithine residues, are water-soluble and exhibit very low CAC values, between 21.6 and 64.2 μM (Table 1).

The lipopeptides form complexes with the CpG-ODN, as shown by the disappearance of the plasmid DNA fluorescence [10], this process is more efficient at pH 4.3 (Fig. 1B) than a pH 7.4 (Fig. 1A), and not affected by the addition of BSA. Complexation of CpG-ODN with 400 μM of lipopeptides was efficient to protect it from DNAse degradation. Representative results obtained for AG2-C16 are shown in Fig. 1C.

To assess the effect of the sequence of lipopeptide on their adjuvant ability, an immunization schedule was performed using the three synthesized compounds: AG2-C16, AG2-C18, or AG3-C16.

Anti-BSA IgG levels started to increase after the second dose (Fig. 2A). Ten days after the third dose, mice receiving BSA+CpG-ODN together with lipopeptides exhibited the highest anti-BSA levels. Among them, the group immunized with AG2-C16+BSA+CpG-ODN had a significant increase in antibodies compared to mice receiving AG2-C16+CpG-ODN, BSA, or BSA+CpG-ODN ($p<0.05$) (Fig. 2B). Moreover, the three formulations containing lipopeptide+BSA+CpG-ODN generated both IgG1 and IgG2a (Fig. 2C). The formulations containing AG2-C16+BSA, either with or without CpG-ODN, induced significantly higher levels of specific IgG1 than the control without adjuvant. On the other hand, AG3-C18+BSA+CpG-ODN and AG2-C16+BSA+CpG-ODN induced significantly higher levels of IgG2a than the same formulations without CpG-ODN (Fig. 2C). No local adverse events were observed with any of the formulations.

Taken together, these results show that lipopeptides improve the immunostimulant activity of PO-CpG-ODN. The AG2-C16+BSA+CpG-ODN formulation was the most efficient in the generation of specific IgG type antibodies, and it was able to induce the production of IgG1 and IgG2a.

Monomeric lipopeptides are versatile molecules able to transport and deliver a great variety of compounds. However, their incorporation as vaccine adjuvant has not yet been explored.

The cationic monomeric lipopeptides designed and ob-

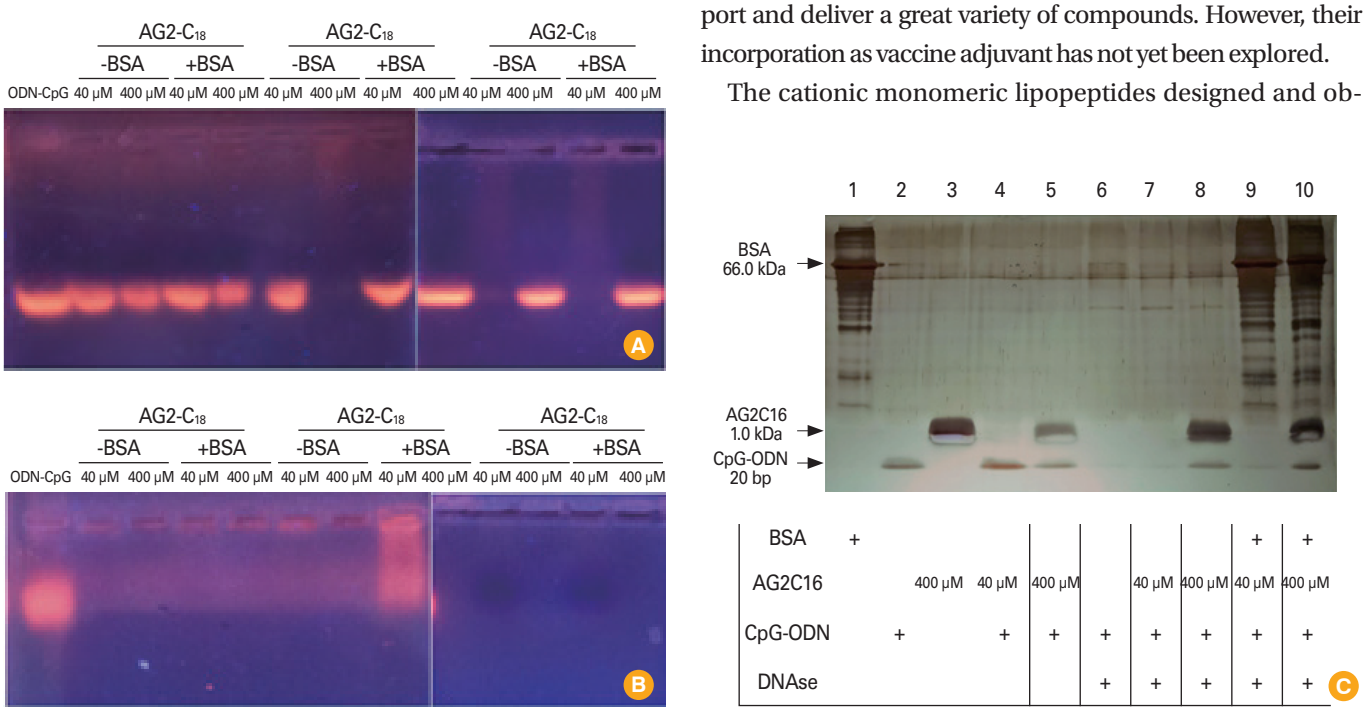

Fig. 1. Complexation of CpG-ODN and lipopeptide assessed by electrophoresis in 2% agarose gel. Lipopeptides+CpG-ODN with and without the addition of BSA in phosphate-buffered saline buffer (pH=7.4) (A) or sodium acetate buffer (pH=4.3) (B) were analysed. Three lipopeptides (AG2-C16, AG2-C18, and AG3-C18) in two different concentrations, 40 μM and 400 μM, were evaluated, keeping the concentration of CpG-ODN invariable (7.5 μM), with and without the addition of BSA (100 μg/mL). The capacity of AG2-C16+CpG-ODN complexes to protect the CpG-ODN from DNase degradation was assessed and results were analysed by 15% SDS-PAGE and Silver stained (C). The RQ1 DNase digestion capacity was demonstrated by the treatment of free CpG-ODN (line 6). BSA in the formulation did not affect the CpG-ODN protection (lines 9 and 10). CpG-ODN, CpG-oligodeoxynucleotides; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

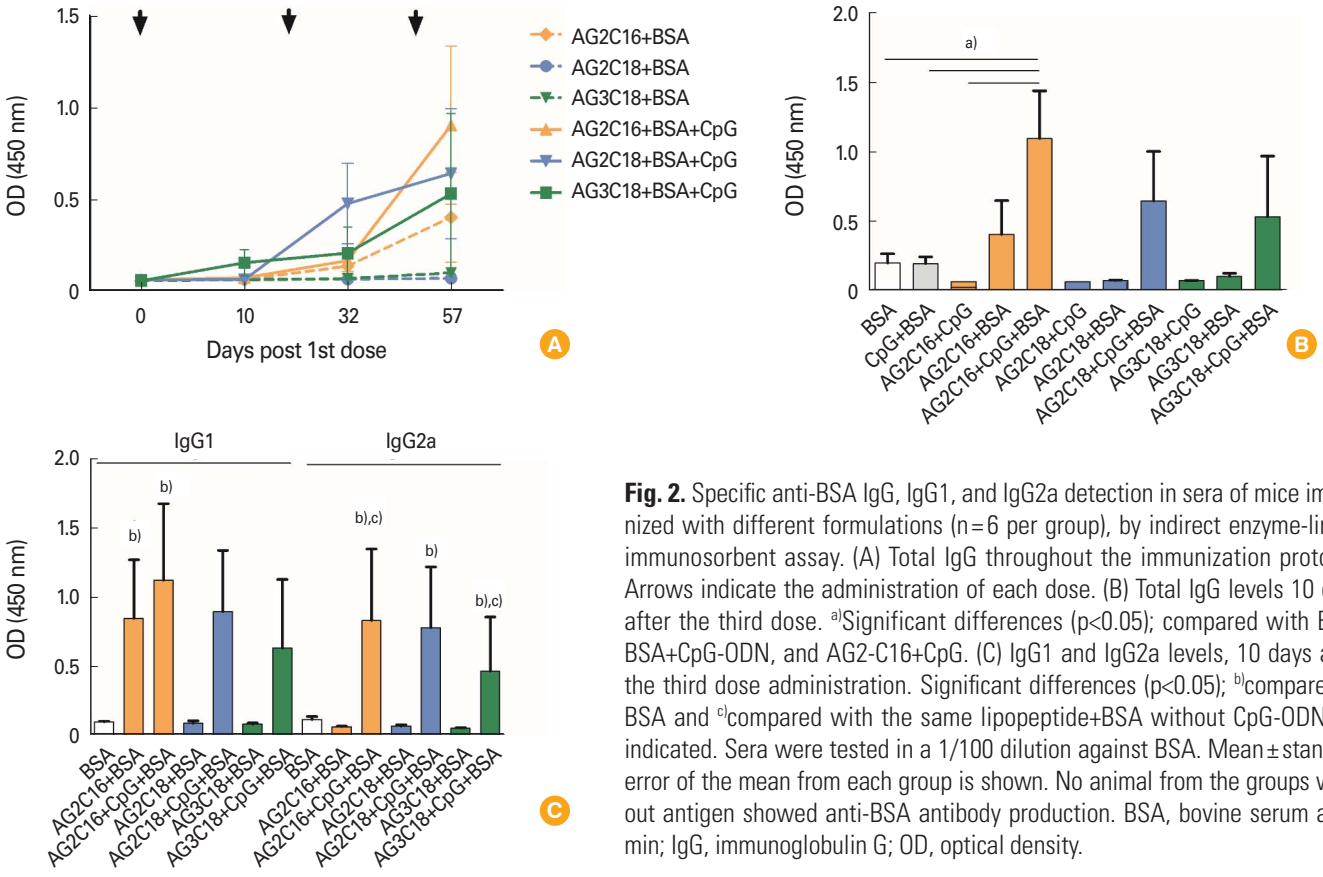

Fig. 2. Specific anti-BSA IgG, IgG1, and IgG2a detection in sera of mice immunized with different formulations (n=6 per group), by indirect enzyme-linked immunosorbent assay. (A) Total IgG throughout the immunization protocol. Arrows indicate the administration of each dose. (B) Total IgG levels 10 days after the third dose. [a]Significant differences ($p<0.05$); compared with BSA, BSA+CpG-ODN, and AG2-C16+CpG. (C) IgG1 and IgG2a levels, 10 days after the third dose administration. Significant differences ($p<0.05$); [b]compared to BSA and [c]compared with the same lipopeptide+BSA without CpG-ODN are indicated. Sera were tested in a 1/100 dilution against BSA. Mean±standard error of the mean from each group is shown. No animal from the groups without antigen showed anti-BSA antibody production. BSA, bovine serum albumin; IgG, immunoglobulin G; OD, optical density.

tained here are water-soluble and have low MW, leading to easy synthesis and handling of these molecules. Moreover, they have a low CAC value compared to those previously reported [11]. The CAC values for AG2-C16 (64 μM) and AG2-C18 (21 μM) decrease as their hydrophobic chains increase in length. The CAC represents the ability of the lipopeptide to form micelles and to associate in complexes with other compounds, such as proteins or nucleic acids. The lower the CAC value, the better the performance of the carrier [12].

We demonstrate here that cationic lipopeptides may be used in subunit vaccine formulations and that they can enhance the humoral immune response induced by a protein antigen. The adjuvant ability depends on the peptide sequence and the length of the hydrophobic chain. Among the molecules tested in this study, AG2-C16 was the most active. This lipopeptide showed adjuvant ability not only in the presence of CpG-ODN but also without the immunostimulant, suggesting that it provides, at least partially, an adjuvant effect by its own, possibly with a Th2 biased response. Nevertheless, when analysing the antibody levels induced by the different combinations, it is evident that the lipopeptide enhances the immunostimulant effect of the CpG-ODN.

The CpG-ODN used in this study has natural phosphodiester bonds, and it did not show any adjuvant activity when combined with BSA, as it was previously observed by others [2,13,14]. On the contrary, the addition of lipopeptides improved the response. This effect correlates well with the ability of these lipopeptides to transport DNA and to protect it from degradation, as previously reported and demonstrated here for our molecules [15].

Adjuvant ability was demonstrated by other types of lipopeptides, such as peptides representing epitopes, that are covalently linked to hydrophobic chains, leading to antigenic compounds with incorporated adjuvant properties [16]. The lipopeptides reported here may be combined with any antigen, becoming a promising platform of adjuvant systems.

In summary, formulations presented here can enhance the immune response against protein antigens, assigning a new role to *gemini* lipopeptides.

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