The formulation of a broadly protective vaccine to prevent the serogroup B Neisseria meningitidis (MenB) disease is still an unmet medical need. We have previously reported the induction of bactericidal and protective antibodies against MenB after immunization of mice with a phage-displayed peptide named 4 L-5. This peptide mimics a capsular polysaccharide (CPS) epitope in MenB. With the aim of developing vaccine formulations that could be used in humans, we evaluate in this study various forms of presentation to the immune system of the 4 L-5 sequence, based on synthetic peptides. We synthesized the following: (i) a linear 4 L-5 peptide, (ii) a multiple antigen peptide containing four copies of the 4 L-5 sequence (named MAP), which was then dimerized, and the product named dimeric MAP, and (iii) a second multiple antigen peptide, in this case with two copies of the 4 L-5 sequence and a copy of a T-helper cell epitope of tetanus toxoid, which was then dimerized and the product named MAP-TT. The linear peptide, the MAP, and the dimeric MAP were conjugated to the carrier protein P64K by different conjugation methods. Plain antigens and antigens coupled to P64K were used to immunize BALB/c mice. Of those variants that gave immunogenic results, MAP-TT rendered the highest levels of specific antipeptide IgG antibodies and serum bactericidal activity. These results can find application in the development of meningococcal vaccine candidates and in peptide-based vaccines strategies.

INTRODUCTION

Recent advances in the field of synthetic peptides have increased interest in their applications as therapeutic or prophylactic formulations. Epitope-based peptide vaccines can be considered the result of the precise selection of vaccine components and allow for accurate direction of immune response. This response is highly specific, almost lacking unspecific binding to molecular structures other than the desired targets. Other advantages of synthetic peptide-based vaccines include the chemical definition, safety, and ease of production and storage.

The fine specificity attained with epitope-based synthetic peptide vaccines is of particular relevance when the same antigen contains both protective and nonprotective or worse, undesirable, epitopes. For example, the capsular polysaccharide (CPS) of Neisseria meningitidis serogroup B (MenB) cells, which is a linear homopolymer of α(2-8)-linked N-acetyl neuraminic acid or polysialic acid (2, 3), has both human cross-reactive and human non-cross-reactive antigenic determinants (4, 5). The discovery of these two classes of epitopes in MenB CPS was facilitated by experiments in which the N-acetyl moieties of MenB CPS were replaced by N-propionyl groups, which is a chemical modification that greatly increases polysaccharide immunogenicity (6, 7).

The identification of peptide mimetics of MenB CPS-specific antigenic determinants is a strategy aimed to further the development of vaccine formulations to cover all meningococcal serogroup B strains. After the screening of a phage-displayed peptide library with the bactericidal and protective monoclonal antibody (mAb) 13D9, raised against N-propionylated (Npr)-MenB CPS (8), we previously identified the sequence named 4 L-5 (RGDKSRPPVWYVEGE) as a peptide mimetic of MenB CPS (9). The phage-displayed 4 L-5 peptide induced antibodies with bactericidal and protective activity against MenB. In the present study, we addressed the design, preparation, and evaluation of the antibody response in mice, of vaccine
formulations based on synthetic peptide structures with the 4 L-5 sequence, compatible with a possible future use in humans.

EXPERIMENTAL PROCEDURES

Chemicals. Boc-protected amino acids, MBHA resin, TBTU, and HOBt were obtained from Bachem (Switzerland). DIC was from Merck (Germany). Peptide synthesis grade DMF, dichloromethane, DIEA, TFA, and HPLC-grade acetonitrile were from Caledon (Canada). The water used was from the Milli-Q Ultrapure Water system (Millipore, Billerica, MA). All other reagents were of the highest quality available commercially.

Peptide Synthesis. The 4 L-5 sequence (RGDKSRPPVYWEGE) was synthesized as a linear simple peptide, as well as multiple antigen peptides (MAP) (10), as shown in Figure 1. The MAP-TT also included the T-helper cell epitope P2 from tetanus toxoid (TT, residues 830-844, QYIKANSKFIGITEL) (11) (P2-TT). Synthesis was performed manually on MBHA resin by a stepwise solid-phase procedure using the Boc/Benzyl strategy (12). All side-chain functions were protected with HF labile groups. The Lys residue responsible for ramification was introduced as Boc-Lys(Boc)OH and coupled with TBTU/DIEA.

Amino acids (aa) were coupled using DIC/HOBt or TBTU/HOBt/DIEA activation, and completion of the coupling reaction was monitored by the ninhydrin test (13). Side-chain deprotection and cleavage from the solid support was performed following the “Low-High” HF procedure (14) with HF:dimethylsulfide-p-cresol (25:65:10 v/v) for 2 h at 0 °C and HF-dimethylsulfide-anisole-thiocresol (79:8:10:0.2 v/v) for 1 h at 0 °C. The peptide was extracted with 30% HOAc in H₂O and freeze-dried.

Analysis and Purification. Peptides were analyzed by HPLC system AKTA 100 (GE Healthcare, USA). Analytical separation was achieved in a reverse-phase (RP) C18 column (Vydac, 4.6 × 150 mm, 5 μm). A linear gradient from 5% to 60% of solvent B over 35 min at a flow rate of 0.8 mL/min was used. The preparative purification was performed on the HPLC system LaChrom (Merck Hitachi, Germany). Separation was achieved by RP C18 column (Vydac, 25 × 250 mm, 25 μm). A linear gradient from 15% to 45% of solvent B over 50 min at a flow rate of 5 mL/min was used. Detection was accomplished at 226 nm. Solvent A: 0.1% (v/v) TFA in water. Solvent B: 0.05% (v/v) TFA in acetonitrile. The purified peptides were characterized for identity by electrospray ionization mass spectrometry (ESI-MS) in a hybrid quadrupole–time-of-flight instrument (Q-TOF1, Waters, USA) fitted with a nanospray ion source.

Dimerizations. The purified monomer was dissolved in water at 4 mg/mL, and the pH was adjusted to 8–9 with 25% ammonium hydroxide (15). The reaction mixture was stirred at room temperature, and the progress of the oxidation reaction was monitored by the Ellman test (16). Dimerization was confirmed by RP-HPLC and ESI-MS analysis. Dimers were purified by RP-HPLC.

Conjugations. Carboximidide Method (CDI Method). The P64K carrier protein (17, 18) was highly purified under GMP conditions, at the Production Unit of the Center for Genetic Engineering and Biotechnology, Havana, Cuba (CIGB). P64K protein (5 mg) was dissolved in 1 mL of phosphate-buffered saline (PBS) pH 7.2, and the pH was adjusted to 5 with a 0.1 mol/L HCl. EDC (10 mg) was then added as bioconjugation reagent (19), and the reaction mixture was stirred for 30 min at 4 °C. Free EDC was separated by gel filtration on a PD10 column equilibrated with PBS pH 7.2. Next, 5 mg of peptide dissolved in PBS pH 7.2 was added to the activated protein, and the reaction mixture was stirred for 3 h at room temperature. The free peptide was separated from the peptide-P64K conjugate by dialysis against PBS pH 7.2 at 4 °C.

Succinic Anhydride-CDI Method (Succ-CDI Method). P64K protein (5 mg) was dissolved in 1 mL of phosphate buffer pH 8.0, and 0.5 mg of succinic anhydride was added as reported (20). The reaction mixture was stirred for 30 min at room temperature, and free succinic anhydride was removed by gel filtration on a PD10 column equilibrated with PBS pH 5. Addition of EDC and subsequent steps were as described in the CDI method, except that incubation with EDC lasted only 10 min.

MPS Method. P64K protein (5 mg) was dissolved in 0.5 mL of phosphate buffer pH 6.0 and 0.1 mL of a solution of the heterobifunctional bioconjugation reagent MPS (19) at 10 mg/mL in DMF was added. The reaction mixture was stirred for 30 min at room temperature. Free MPS was separated by gel filtration on a PD10 column equilibrated in phosphate buffer pH 6. Then, 5 mg of peptide dissolved in phosphate buffer pH 6 was added to the activated protein, and the reaction mixture was stirred for 3 h at room temperature. The free peptide was separated by dialysis against PBS solution pH 7.2 at 4 °C.

Amino Acid Analysis. Amino acid analyses were performed by the laboratory of Dr. Fernando Albericio at the Barcelona Scientific Park, Barcelona, Spain. Samples were hydrolyzed with 6 M HCl for 1 h at 155 °C in vacuum-sealed ampules. After evaporation of the acid, the hydrolyzed sample was dissolved in loading buffer. The chromatographic separation of free aa was done with an automatic analyzer Beckman 6300 (Beckman Instruments Inc., USA), operated following the manufacturer’s instructions and with detection by the ninhydrin reaction (21).

Bacterial Strains and Growth Conditions. The N. meningitidis strain CU385 (B:4:19.15; ST = 33) (22, 23) was obtained from the collection of the Finlay Institute (Havana, Cuba). It was grown for 18 h at 37 °C on a candle jar on Chocolate agar plates (Brain and Heart Infusion (Difco, USA) agar base with 7.5% (v/v) defibrinated goat blood heated under stirring at 80 °C until color change from red to brown). A fresh agar plate was streaked and similarly cultured for 4 h. Agar plates were supplemented with vancomycin/colistin/nystatin (Oxoid, England) following the manufacturer’s instructions.

Monoclonal Antibodies. The IgG₃ mAb 13D9 was specifically raised against Npr-MenB CPS. This antibody has bactericidal activity against MenB and is protective in an infant rat model (8). The antibody was a generous gift from Dr. Harold Jennings from the National Research Council of Canada. The mAb 114, from the collection of the CIGB, recognizes the sequence DAADIKTIHP of P64K protein from N. meningitidis (24).

Protein Concentration Determination. The protein concentration was determined using the Coomassie reagent method (25).

Mice Immunizations. Groups of 6 female BALB/c mice (H-2d), obtained from the Center for Production of Laboratory Animals (CENPALAB, Havana, Cuba), aged 7–8 weeks, received four weekly intraperitoneal injections of the antigens.
Table 1. Antigens Used for Immunizations

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Description</th>
<th>MW&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peptide/protein ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Molar concentration of L/4 L-5 sequence in vaccine formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>P64K</td>
<td>P64K carrier protein</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L/4 L-5</td>
<td>Linear peptide consisting only of the 4 L-5 sequence</td>
<td>1772.89</td>
<td>—</td>
<td>1.41 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>L/4 L-5-P64K-CDI</td>
<td>L/4 L-5 conjugated to P64K protein by the carbodiimide (CDI) method</td>
<td>—</td>
<td>1.92</td>
<td>7.12 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAP</td>
<td>Four-arm multiple antigen peptide containing four copies of the 4 L-5 sequence with a cysteine residue added to the C-terminus for further conjugation purposes or dimerization</td>
<td>7603.53</td>
<td>—</td>
<td>1.32 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>D/MAP</td>
<td>8-arm dimeric MAP containing 8 copies of the 4 L-5 sequence resulting from the disulfide dimerization of MAP</td>
<td>15205.05</td>
<td>—</td>
<td>1.32 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAP-P64K-CDI</td>
<td>MAP conjugated to P64K using the CDI method</td>
<td>—</td>
<td>1.64</td>
<td>2.14 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAP-P64K-Succ-CDI</td>
<td>MAP conjugated to P64K treated with succinic anhydride before conjugation by the CDI method</td>
<td>—</td>
<td>5.12</td>
<td>4.97 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAP-P64K-MPS</td>
<td>MAP conjugated to P64K using maleimide propionic acid N-hydroxysuccinimide ester (MPS) as coupling agent</td>
<td>—</td>
<td>4.58</td>
<td>4.63 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>D/MAP-P64K-CDI</td>
<td>D/MAP conjugated to P64K using the CDI method</td>
<td>—</td>
<td>0.99</td>
<td>2.50 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAP-TT</td>
<td>Four-arm multiple antigen peptide, which is a disulfide dimer, with monomers consisting of two copies of L/4 L-5 connected by a lysine branching point to the T-helper cell epitope P2-TT from tetanus toxoid, with one cysteine residue added at the C-terminus where dimerization takes place</td>
<td>11192.70 (5593.86&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>—</td>
<td>8.93 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Molecular weight in Daltons. <sup>b</sup> Molecular weights calculated and found for the monomer specie. <sup>c</sup> Peptide to protein ratio for P64K conjugates as determined by amino acid analysis.

at the doses indicated below. The antigens were emulsified with Complete Freund Adjuvant in the first injection and Incomplete Freund Adjuvant in successive ones. The final volume of each injection was 0.2 mL. Serum samples were drawn before each immunization and a week after the last one. Sera were stored at −20 °C until use. All animal experimentation was carried out in compliance with the legal requirements of the national authority. For immunizations with synthetic antigens based on tetanus toxoid, with one group received 5, 25, or 50 μg of each of the antigens listed in Table 1. For the dose response study with MAP-TT, animals received 5, 25, or 50 μg of MAP-TT and one group received 50 μg of P64K protein.

ELISA. ELISA of Antigens. The volume of the assay was 50 μL per well. All antigens listed in Table 1, previously diluted at 10 μg/mL in 0.05 M sodium carbonate buffer pH 9.6, were directly applied onto high-binding polystyrene 96-well flat-bottomed microtiter plates (Costar, Cambridge, USA) and incubated for 18 h at 4 °C. Plates were washed thrice with PBS/0.05% (v/v) Tween 20 (PBS/T) and blocked with skimmed milk at 5% (w/v) in PBS (blocking solution). The mAb 13D9 was diluted to 5 μg/mL in blocking solution and added for 2 h at 25 °C. After repeating the washes, bound antibodies were detected with HRP-conjugated goat antiguinea IgG antibodies (Sigma, USA), with H<sub>2</sub>O<sub>2</sub> and the chromogen o-phenylenediamine. The reaction was stopped with sulfuric acid 2.5 N, and absorbance was read at 492 nm (A<sub>492 nm</sub>) in an ELISA plate reader (SensiDent Scan, Merck, Germany). All samples were analyzed in duplicate, and results were expressed as mean A<sub>492 nm</sub> values. A signal was considered positive when surpassed twice the signal obtained for the negative control antigen (P64K protein).

Serum ELISA. The steps followed were basically as in the ELISA of antigens. Plates were coated for 18 h at 4 °C with D/MAP at 10 μg/mL in 0.05 M sodium carbonate buffer, pH 9.6. After blocking, individual serum samples obtained on day 0 (preimmune) and one week after immunizations, were diluted 1:200 in blocking solution and added to plates for 2 h at 25 °C. Bound IgG antibodies were detected with HRP-conjugated goat antiguinea IgG antibodies (Sigma, USA). All serum samples were analyzed in duplicate and mean values were used for analysis. Results were expressed as the ratio of ELISA A<sub>492 nm</sub> read for immune sera/A<sub>492 nm</sub> read for preimmune sera. A ratio of ≥2 was chosen as the cutoff value to consider the response as positive.
reactivity that was mostly accounted for by IgM, results are directly
expressed as $A_{492 \text{ nm}}$. For comparison between levels of
IgG and IgM, IgG was reassessed but using pooled sera from
each group of mice.

**Serum Bactericidal Activity Assay.** Pooled serum samples
from each group were assayed for SBA against the *N. meningitidis*
strain CU385, following basically the assay of Maslanka
(26) with some modifications previously described (9). Twofold
serial dilutions of preimmune and immune sera, starting at 1:8,
were assayed. Titters of SBA were expressed as the reciprocal
of the final serum dilution giving $50\%$ killing of the inoculum
after 60 min of bactericidal reaction. Sera with SBA titers of
$\geq 8$ were considered positive.

**Statistical Analysis.** All data sets, after transformation
(Log$_{10}$), passed the normality test (Kolmogorov
after 60 min of bactericidal reaction. Sera with SBA titers of
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coated with D/MAP. Sera were diluted 1:200. Mean the first immunization was measured after two (connector line), an increase in the IgG levels with respect to the first immunization was measured after two (connector line). No differences were detected in the IgG levels between the second and third immunizations (P > 0.05).

For the groups immunized with D/MAP, MAP-P64K-Succ-CDI, and MAP-P64K-MPS (Figure 4, black solid connector lines), an increase in the IgG levels with respect to the first immunization was observed after two (P < 0.01 for D/MAP and P < 0.001 for MAP-P64K-Succ-CDI and MAP-P64K-MPS), three (P < 0.001 for all groups), and four injections (P < 0.001 for all groups). No differences were detected in the IgG levels of any of these groups between the second and third immunizations (P > 0.05).

For the group immunized with MAP-TT (Figure 4, dashed connector line), an increase in the IgG levels with respect to the first immunization was measured after two (P < 0.01), three comparisons). No differences in the IgG levels were detected between the second and third immunizations (P > 0.05).

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A dashed line represents the assumed seroconversion criterion (A). Dose (one week after three and four injections. No differences were detected in the IgG levels, after both three (P < 0.001) and four injections (P < 0.05). ELISA plates were coated with D/MAP. Sera were diluted 1:200. Mean ± SD of A492 nm values for preimmune sera was 0.085 ± 0.016. The gray dashed line represents the assumed seroconversion criterion (A492 nm immune serum/A492 nm preimmune sera ≥ 2).

Figure 6. Dose response study with the antigen MAP-TT (mean ± 95% CI). Mice (n = 6) received doses of 5, 25, or 50 µg of MAP-TT. Serum samples were obtained from mice on day 0 (preimmune) and one week after the third injection. ELISA plates were coated with D/MAP. Sera were diluted 1:50. Antibody binding was detected using isotype-specific reagents.

The increase in the available T-helper cell epitopes, by means of conjugation to the carrier protein P64K, enhanced the immunogenicity of the MAP structure, as occurred with the conjugates of MAP to P64K obtained by means of Succ-CDI and MPS methods. The conjugation of simple peptides as well as the synthetic peptide-based vaccines offer several advantages over other vaccine forms, particularly with regard to safety and ease of production. However, they also have a number of limitations such as poor immunogenicity in the case of simple peptides and the need to guarantee stimulation of T-helper cell (1). These drawbacks could be overcome using adequate systems of peptide presentation to the immune system.

An approach usually exploited to increase the peptide immunogenicity is the synthesis of branched peptides such as MAPs (10, 31). To provide T-cell help, several strategies could be assayed, such as the conjugation of peptides, including MAPs, to carrier proteins (18, 32, 33). P64K is an extensively characterized N. meningitidis protein antigen (17, 34–36) that had proven immunogenicity and nontoxicity when assayed as a carrier in a phase 1 clinical trial (37).

Another strategy is the synthesis of MAP structures containing in the same construct the desired B-cell epitope and the sequence of a defined T-helper cell epitope (38) such as the P2-TT sequence (11). This is a T-helper cell epitope, not restricted to a single human MHC haplotype (11). Such a promiscuous epitope could serve as a source of T-cell help in an outbred population, which could be helpful in a possible future application in humans of the 4 L-5 sequence. This peptide could also be used to provide T-cell help in murine models, including the BALB/c mouse strain used here.

After immunization of BALB/c mice, the linear 4 L-5 peptide was unable to induce antipeptide antibodies, whereas the unconjugated MAP, both monomeric and dimeric, induced the production of specific antipeptide antibodies. Significantly higher antibody titers have previously been obtained after immunization with MAPs than with the corresponding simple linear peptide (31, 40, 41). The immunogenicity of the MAP structure, in the absence of an exogenous source of T-helper cell epitopes, could indicate the presence of both T and B epitopes in the 4 L-5 sequence, either alone or in combination with the branching residues. This latter case could explain the failure of the linear peptide to induce antipeptide antibodies; alternatively, this failure could be the result of low B- and/or T-cell stimulation, which would be overcome by the multimeric presentation of the peptide sequence in the MAP format. The presence of the four IgG subclasses in sera from mice immunized with MAP and D/MAP could be an indicator of class switching as a result of the recruitment of T-helper cells (42).
as MAPs is generally expected to increase the immunogenicity of the synthetic structures (18, 32, 33). Surprisingly, the immune response against the linear peptide did not increase when conjugated to P64K protein, and inhibition of the response was verified after immunization with the conjugates of D/MAP and MAP obtained by the CDI method. A common characteristic of these three conjugates is that the CDI method was used for their production. The use of carbodiimide as a reagent for conjugation implies that EDC first reacts with available carboxylic groups of the carrier protein, which upon activation can react with a primary amine of the peptide to form an amide bond (19). The P64K protein has the following carboxylic groups: the carboxyl terminus and 38 aspartic and 41 glutamic residues (34). The reaction involves mainly the ε-amine group of the aa in the amine terminal position in the peptide, which is an R in the 4 L-5 sequence (R1GDKSRPPVWYVEGE135). The reaction may also involve the ε-amine group of the side chain in K residues (19, 27), like the K4 in the 4 L-5 sequence. Neither of these 2 aa (R1 or K4) are included within the minimum region of the 4 L-5 sequence involved in direct binding to mAb 13D9, previously mapped in the tetrapeptide WYVE (9). The possibility that peptide binding to the carrier protein by one of these two residues causes the low immunogenicity of this conjugate, while keeping its capacity of binding mAb 13D9, was discarded, since the MAP conjugated to P64K by the Succ-CDI method was immunogenic and this methodology also implies peptide binding to the carrier protein through residues R1 and K4. The difference between the CDI and Succ-CDI methods is that the latter begins with a treatment of the protein with succinic anhydride under mildly alkaline conditions, a process that converts the primary amine groups of the carrier protein to carboxylic acids (27). The free succinic anhydride is removed by dialysis to prevent peptide inactivation and the reaction continues in the same way as in the CDI method. Thus, primary amine groups in the P64K carrier protein are the amine terminal and 40 lysine residues (34); thus, the use of the Succ-CDI method could increase the number of sites available for peptide conjugation and hence the number of peptide molecules bound per molecule of protein, thus changing the amount of peptide administered per dose of total protein injected. Moreover, in the case of the CDI method, it is possible to obtain protein cross-linking after activation with EDC. This cross-linking may compromise sites for peptide conjugation, while the previous treatment of the carrier protein with succinic anhydride under mild alkaline conditions circumvents this problem (27). Also, the application of succinylation methodologies introduces a spacer arm into the carrier protein thereby extending the reactive group away from its surface and thus providing less steric hindrance to conjugation. The use of the heterobifunctional conjugation reagent MPS also involves the introduction of a spacer arm between the peptide and the carrier, while the zero-length cross-linking procedure mediated by the water-soluble carbodiimide EDC implies that no bridging molecule is introduced between the peptide and the carrier (27).

The aa analysis of the conjugates containing the MAP showed that the conjugation efficiencies (peptide/protein molar ratio) of MAP-P64K-Succ-CDI and MAP-P64K-MPS are about 3-fold that of MAP-P64K-CDI, as a consequence L/4 L-5 sequence dosages were lower for the last one. Molar concentrations of the L/4 L-5 sequence in the other two conjugates obtained by the CDI method but with peptides other than MAP (L/4 L-5-P64K-CDI and D/MAP-P64K-CDI) were also lower with regard to the immunogenic conjugates (MAP-P64K-Succ-CDI and MAP-P64K-MPS), due to a lower conjugation efficiency, even though the number of L/4 L-5 sequence in D/MAP peptide is double that of MAP. Differences in immunogenicity observed among conjugates in the present work could be, at least partly, attributable to differences in the effective amount of the L/4 L-5 sequence presented to the immune system. It is well-known that the dose of administered antigen is a key variable leading to elicitation of an immune response or to the induction of tolerance (28). Therefore, for the preparation of particular immunogen conjugates, several antigen-to-carrier ratios and methods of conjugation may need to be investigated (43–45).

MAP-TT elicited the highest levels of specific antipeptide and bactericidal antibodies. This last property is of the highest importance, as the induction of complement-dependent serum bactericidal activity is currently considered as the immunological surrogate of protection against meningococcal disease (46). The lower response against MAP-P64K-Succ-CDI and MAP-P64K-MPS may be due to the lower dosage of the L/4 L-5 sequence in the conjugates with regard to MAP-TT or the different sources of T-helper cell epitopes. While P64K and the tetanus toxoid may act similarly as sources of T-helper cell epitopes (18), a comparison of P64K with only TT epitope has not been performed. Other possibilities are adoption of fewer immunogenic peptide conformations in conjugates or masking of peptide epitopes by P64K. Functional differences in the elicited antibodies might also be caused by the differences observed in IgG subclass composition. IgG2a and IgG2b murine isotypes are the most effective in mediating complement fixation (47). The higher levels of these two subclasses in the response against MAP-TT with respect to MAP-P64K-Succ-CDI and MAP-P64K-MPS could mediate complement-dependent bacterial killing more efficiently, thus possibly accounting for the higher serum bactericidal responses detected.

Fully synthetic structures, such as the MAP-TT, containing both defined B- and T-helper cell epitopes, are advantageous over peptide–protein conjugates. Their composition is easily characterized (1), and there is lower batch to batch variability in a productive process (48). Also, the use of carrier molecules is associated with the suppression of the antipeptide antibody response, and there are concerns about the dose that can be safely administered (49, 50).

The antigenicity and immunogenicity of a peptide mimotope could vary by changing the molecular context in which the peptide is first identified (51). However, the sequence 4 L-5 was still able to bind the mAb 13D9 or to elicit antibodies with bactericidal activity against N. meningitidis serogroup B when taken from the filamentous phage context, where this mimotope was first identified (9).

Our data emphasize the paramount importance of the presentation form of a peptide sequence to the immune system. Since immunogenicity depends on many complex interactions with various elements of the host immune system, it is still not possible to predict the peptide construct that will be the most effective immunogen. It is therefore important to compare several forms of antigen presentation. The results presented here can find application in the development of meningococcal vaccine candidates and in peptide-based vaccines strategies in general.

**ACKNOWLEDGMENT**

The authors thank Dr. Harold Jennings from the National Research Council of Canada for providing mAb 13D9. The authors also thank colleagues from the Finlay Institute (Havana, Cuba) for providing the meningococcal serogroup B strain CU385 and colleagues from the Animal Care Unit (CIGB) for animal care and handling. We also thank Dr. Juan Arrieta (CIGB) for critical reading of the manuscript. The work carried out in Barcelona was partially supported by CICYT (CTQ2009-07758) and the Generalitat de Catalunya (2009SGR 1024).
REFERENCES


BC100299G