RESEARCH

Antibodies elicited by immunization with cyclic synthetic peptides comprising the entire sequence of meningococcal PorA loop 1 subtype P1.19 recognize the outer membranes of Neisseria meningitidis

✉ Hilda E Garay[1], Tamara Menéndez[2], Maité Delgado[2], Ricardo Silva[2], Osvaldo Reyes[1]

[1]Química-Física Division
[2]Vaccines Division
Center for Genetic Engineering and Biotechnology (CIGB)
Ave. 31 e/ 158 y 190, Cubanacán, Playa, POBox 6162, CP 10600,
Ciudad de La Habana, Cuba
E-mail: hilda.garay@cigb.edu.cu

ABSTRACT

The possibility was studied of eliciting antibodies that are able to recognize *Neisseria meningitidis* outer membranes by immunizing with synthetic peptides containing the sequence of the exposed loop 1 (variable region 1 or VR1) subtype P1.19 from meningococcal PorA protein. It has been shown that the antibodies elicited by immunization with a cyclic synthetic peptide containing the apex amino acids of the PorA P1.19, fail to recognize the native PorA protein. In the current study we tested a cyclic synthetic peptide containing the complete sequence of meningococcal PorA VR1 P1.19. Specific anti-PorA antibodies were induced by the immunization of Balb/C mice with unconjugated and BSA-conjugated peptides. The elicited antibodies were able to react with the native PorA protein as presented in the meningococcal outer membrane vesicles. These results highlight the importance of presenting the VR1 to the immune system as close as possible to the natural conditions in order to obtain an adequate immune response.

Keywords: PorA, synthetic peptide, solid phase peptide synthesis, conjugated peptide, meningococci, conformation

Biotecnología Aplicada 2008;25:242-246

RESUMEN

Los anticuerpos inducidos contra péptidos sintéticos correspondientes con la secuencia entera del lazo 1 de PorA del subtipo P1.19 del meningococo reconocen las membranas externas de *Neisseria meningitidis*. La posibilidad de obtener anticuerpos capaces de reconocer la membrana externa de *Neisseria meningitidis* se estudió mediante la inmunización con péptidos sintéticos que contienen la secuencia del lazo expuesto 1 (región variable 1 o RV1) del subtipo P1.19 de la proteína PorA del meningococo. Ha sido demostrado previamente que los anticuerpos inducidos por la inmunización con un péptido sintético cíclico que comprende los aminoácidos del ápice del lazo 1 del subtipo P1.19 de PorA no reconocen la proteína nativa. En el presente trabajo se evaluó un péptido sintético cíclico que comprende la secuencia completa de la RV1 de PorA. Anticuerpos específicos anti-PorA fueron inducidos por la inmunización de ratones Balb/C con el péptido conjugado a BSA y no conjugado. Los anticuerpos inducidos reaccionaron con la proteína PorA nativa, tal como se presenta en vesículas de la membrana externa de la bacteria. Estos resultados destacan la importancia de presentar la RV1 al sistema inmune tan similar como sea posible a la natural para obtener una respuesta inmune adecuada.

Palabras clave: PorA, péptidos sintéticos, síntesis en fase sólida, péptidos conjugados, meningococo, conformación

*I*ntroduction

Bactericidal antibodies against the PorA outer membrane porin protein from *Neisseria meningitidis* play a major role in the protection against meningococcal disease [1, 2]. A two-dimensional model of PorA protein predicts the presence of eight cell surface-exposed loops in the protein [3]. Sequence comparison of the porA gene and the deduced amino acid sequence from different serosubtypes reveals the divergence between strains that are confined largely to two discrete variable regions, designated variable region 1 (VR1) and variable region 2 (VR2), located at the apices of exposed loops 1 and 4, respectively, thus generating two separate serosubtypes in each strain [4]. The PorA protein of the most frequent clinical isolate in Cuba is of the serological classification B:4:P1.19,15 [5], thus having a P1.19 determinant in loop 1 (VR1) and a P1.15 determinant in loop 4 (VR2) [6]. The CU385 strain, with the same serological classification, is the Cuban reference strain [7]. The exposed loops 1 and 4 are the targets of the bactericidal antibodies against the protein [1].

Previous studies have demonstrated that the immunization of laboratory animals with synthetic peptides, derived from surface loop 4 of PorA protein of subtypes P1.13, P1.15, P1.16a and P1.16b, produced antisera that can to bind the native PorA protein on the surface of meningococci [8-14]. The studies with the loop 4 peptide corresponding to subtype P1.15, conducted by us, included the immunization with polymers of the cyclic peptide [10], conjugates to the

1. Saukkonen K, Leinonen M, Abdillahi H, Poolman JT. Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and *in vitro* bactericidal assay. Vaccine 1989;7:325-8.

2. Idanpaan-Heikkila I, Hoiby EA, Chattopadhyay P, Airaksinen U, Michaelsen TM, Wedege E. Antibodies to meningococcal class 1 outer-membrane protein and its variable regions in patients with systemic meningococcal disease. J Med Microbiol 1995;43:335-43.

3. van der LP, Heckels JE, Virji M, Hoogerhout P, Poolman JT. Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect Immun 1991;59:2963-71.

✉ Corresponding author

P64K carrier protein of linear and cyclic peptides [11] and the phage-displayed peptide [12]. However, the sequences corresponding to the subtypes associated with loop 1 of PorA protein [15, 16], have not been evaluated as immunogens in the form of synthetic peptides, with the exception of our previous work with the sequence corresponding to the VR1 of PorA from the Cuban strain (subtype P1.19) [11]. We immunized mice with a cyclic peptide conjugated to the P64K protein, and although specific anti-peptide antibodies were detected, it was not possible to demonstrate antibody binding to outer membrane vesicles (OMV) extracted from *N. meningitidis* [11]. In this report [11], the sequence of the VR1 contains only the apex amino acids of PorA loop 1, which probably account for the failure of antibodies to recognize the native protein. In the present study we tested the ability of antibodies against a cyclic peptide containing the entire sequence of loop 1 of subtype P1.19 to induce antibodies that can recognize the native PorA protein as presented in the outer membrane of *N. meningitidis*.

Materials and methods

Meningococcal strains and growing conditions

The meningococcal strain CU385 (B:4,7:P1.19,15; ST = 33) [7] was obtained from the *N. meningitidis* strain collection of the Finlay Institute, Havana, Cuba and was grown in Brain and Heart Infusion agar for 18 h at 37 °C on a candle jar.

Outer membrane vesicle preparations

Outer membrane vesicles from *N. meningitidis* strain CU385 were obtained by the LiCl procedure [17]. They were stored at 4 °C.

Protein concentration determination

Protein concentration was determined by the Lowry method [18].

Peptide synthesis and conjugation

Two peptides with sequences corresponding with the exposed loops 1 (sequence: AcCNFQLQLTEPPSKSQ PQVKVTKAKSRICβAlaG) and 4 (sequence: AcC SKSAYTPAHYTRQNNADVFVPAVVGCβAlaAG) of the PorA protein from the Cuban strain CU385 [6] were synthesized using the Boc/Bzl (t-butyloxy-carbonyl/benzyl) strategy [19] on 60 mg of PAM-resin (substitution level 1 mmol/g). Cleavage of the Boc group was carried out with 37.5% trifluoroacetic acid in dichloromethane for 30 min and neutralized with 5% N,N-diisopropylethylamine in dichloromethane three times for 2 min each. The amino acids were coupled using N,N-diisopropylcarbodiimide and the reaction was monitored by the ninhydrin test. The peptides were acetylated at the N-terminal with acetic anhydride. The side-chain was left unprotected and cleavage of the resin was performed following the 'Low-High' hydrogen fluoride (HF) procedure [20] with HF-dimethylsulfide -*p*-cresol (25: 65: 10) for 2 h at 0 °C and HF- dimethylsulfide -anisole-thio-cresol (79.8: 10: 10: 0.2) for 1 h at 0 °C. The peptides were extracted with 30% acetic acid in water and lyophilized.

The peptides were reduced with 100 mM dithiotreitol in 6 mol/L guanidium chloride, 100 mM Tris at pH 8.2 for 2 h at 37 °C and purified by the HPLC system (Pharmacia-LKB), using a RP-C18 (25 x 250 mm, Vydac) column with a linear acetonitrile/water gradient of 15 to 45% for 50 min at a flow rate of 4 mL/min. The absorbance was monitored at 226 nm. The oxidation of thiol groups was carried out with 20% dimethylsulfoxide in water [21]. The peptides were dissolved in acetic acid: H_2O (1:19) at 0.2 mmol/L and pH was adjusted to 6 with 25% ammonium hydroxide. The oxidation reaction was monitored by the Ellman test [22]. The cyclic peptides were analyzed using an analytical column RP-C18 (4.6 x 150 mm, Vydac) with a linear acetonitrile / water gradient from 0 to 60% for 40 min at a flow rate of 0.8 mL/ min. Absorbance was monitored at 226 nm. The cyclic peptides were also studied by matrix-assisted laser desorption ionization-mass spectrometry.

To prepare the conjugates, the cyclic peptides (1 mg) were dissolved in 700 µL of PBS solution pH 7.2, 5 mg of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride were added, the pH was adjusted to 5 with a 0.1 mol/L HCl solution and the reaction was stirred for 10 min. Then, 1 mg of BSA in 300 µL of PBS was added, the pH was adjusted to 6-7 with a 0.1 mol/L NaOH solution and the reaction was stirred for 2 h. The conjugated peptides were purified by gel filtration on a PD10 column [23].

Peptides were named as follows: cVR1 (unconjugated cyclic VR1), BSA-cVR1 (BSA-conjugated cyclic VR1), cVR2 (unconjugated cyclic VR2) and BSA-cVR2 (BSA-conjugated cyclic VR2).

Conjugate analysis by immunoblotting

For the analysis by immunoblotting of BSA-conjugates, 5 µg of OMV preparations from *N. meningitidis* strain CU385, 3 µg of BSA and 20 uL of BSA-conjugated peptides per lane, were suspended in Laemmli sample buffer and subjected to 12.5% SDS-PAGE [24]. Proteins were transferred to 0.45 µm pore size nitrocellulose membranes [25]. The nitro-cellulose sheets were blocked with skimmed milk 5% (v/v) in PBS/ 0.05% (v/v) Tween 20 (Sigma, USA) (blocking solution) and incubated for 2h at RT with specific monoclonal antibodies for the PorA subtypes P1.19 [26] or P1.15 (paper pending publication). Immunological reactivity was detected with horseradish peroxidase-conjugated anti-mouse IgG antibodies (Sigma, USA) and substrate solution containing 3-amino-9-ethylcarbazole and hydrogen peroxide.

Mice immunizations

Groups of 8 Balb/c mice each were inoculated subcutaneously with three doses of 50 µg, every two weeks, with the unconjugated and BSA-conjugated loop 1 and loop 4 peptides. An additional group was immunized with the BSA protein. Preimmune sera and sera after the third dose were extracted and stored at -20 °C until use.

ELISAs

High binding polystyrene 96-well flat-bottomed microtiter plates (Costar, Cambridge, USA) were coated overnight at 4 °C, with either OMV extracted from

4. McGuinness B, Barlow AK, Clarke IN, Farley JE, Anilionis A, Poolman JT, *et al*. Deduced amino acid sequences of class 1 protein (PorA) from three strains of *Neisseria meningitidis*. Synthetic peptides define the epitopes responsible for serosubtype specificity. J Exp Med 1990;171:1871-82.

5. Valcarcel M, Rodríguez R, Terry H. La enfermedad meningococcica en Cuba. Cronología de una epidemia. La Habana, Cuba: Editorial Ciencias Médicas, 1991.

6. Guillén G, Alvarez A, Niebla O, Silva R, González S, Musacchio A, *et al*. Cloning and expression of the porA gene of *Neisseria meningitidis* strain B:4.P1.15 in *Escherichia coli*. Preliminary characterization of the recombinant polypeptide. Acta Biotechnol 1996;16:165-73.

7. Sierra GV, Campa HC, Varcacel NM, Garcia IL, Izquierdo PL, Sotolongo PF, *et al*. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NIPH Ann 1991;14:195-207.

8. Christodoulides M, Heckels JE. Immunization with a multiple antigen peptide containing defined B- and T-cell epitopes: production of bactericidal antibodies against group B *Neisseria meningitidis*. Microbiology 1994;140 (Pt 11):2951-60.

9. Hoogerhout P, Donders EM, van Gaans-van den Brink JA, Kuipers B, Brugghe HF, van Unen LM, *et al*. Conjugates of synthetic cyclic peptides elicit bactericidal antibodies against a conformational epitope on a class 1 outer membrane protein of *Neisseria meningitidis*. Infect Immun 1995;63: 3473-8.

10. Garay H, Niebla O, González LJ, Menéndez T, Cruz LJ, Reyes O. Disulfide bond polymerization of a cyclic peptide derived from the surface loop 4 of class 1 OMP of *Neisseria meningitidis*. Lett Pept Sci 2000;7:97-105.

11. Sardiñas G, González S, Garay HE, Nazabal C, Reyes O, Silva R. Anti-PorA antibodies elicited by immunization with peptides conjugated to P64k. Biochem Biophys Res Commun 2000;277:51-4.

12. Menéndez T, De H, I, Delgado M, Garay H, Martin A, Vispo NS. Immunisation with phage-displayed variable region 2 from meningococcal PorA outer membrane protein induces bactericidal antibodies against *Neisseria meningitidis*. Immunol Lett 2001;78:143-8.

13. Zhu D, Williams JN, Rice J, Stevenson FK, Heckels JE, Christodoulides M. A DNA fusion vaccine induces bactericidal antibodies to a peptide epitope from the PorA porin of *Neisseria meningitidis*. Infect Immun 2008;76:334-8.

14. Jiang J, Abu-Shilbayeh L, Rao VB. Display of a PorA peptide from Neisseria meningitidis on the bacteriophage T4 capsid surface. Infect Immun 1997;65: 4770-7.

15. Sacchi CT, Lemos AP, Brandt ME, Whitney AM, Melles CE, Solari CA, *et al*. Proposed standardization of *Neisseria meningitidis* PorA variable-region typing nomenclature. Clin Diagn Lab Immunol 1998;5:845-55.

16. Feavers IM, Fox AJ, Gray S, Jones DM, Maiden MC. Antigenic diversity of meningococcal outer membrane protein PorA has implications for epidemiological analysis and vaccine design. Clin Diagn Lab Immunol 1996;3:444-50.

meningococcal strain CU385 or the cyclic synthetic peptides corresponding to VR1 (subtype 19) and VR2 (subtype 15) of PorA protein diluted at 10 µg/mL in 0.05 M Sodium-carbonate buffer, pH 9.6. Plates were blocked with the blocking solution. Serum samples were diluted 1/200 in the blocking solution. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Sigma, USA), with the chromogen orthophenylenediamine and the enzyme substrate hydrogen peroxide. Absorbance was read at 492nm (A_{492nm}). All sera were analyzed in duplicate and the average values were used for the analysis.

Statistical analysis:

Data were transformed (Log 10) for the statistical comparisons of differences between groups. Transformed data passed the normality test (Kolmogorov-Smirnov with Dallal-Wilkinson Lillie for the P value) and showed variance homogeneity (Bartlett´s test). The transformed data were analyzed by a parametric ANOVA and the means compared by the Newman-Keuls Multiple Comparison Test. P values < 0.05 were considered significant. GraphPad Prism statistical software (San Diego, CA, USA) was used.

Results and discussion

The importance of the VRs of PorA protein for immunity against *N. meningitidis* has been well established: the VRs are the target for the bactericidal and protective antibodies directed against PorA [1, 2, 27-29].

Several groups have studied the immunogenicity of synthetic peptides with sequences corresponding to the PorA VRs [8-14]. The immunogenicity of peptides, with the sequences of the VRs of PorA of the Cuban strain CU385 (VR1: P1.19 and VR2: P1.15), and the capacity of the induced antibodies to recognize the *N. meningitidis* surface, have also been studied [10-12].

In a previous study [11] we immunized mice with the sequence of the VR1, but it only contained the apex amino acids of loop 1 and the induced antibodies failed to recognize the native protein. Here we tested the capacity of antibodies elicited against the entire loop 1 to recognize the native PorA protein. For the control of the experiments we used the peptide corresponding to the VR2 (subtype P1.15).

The peptides were synthesized on a solid phase following the Boc/Bzl strategy and restricted by cyclization to mimic the conformation of the surface loops. PAM-resin was used for the synthesis of peptides with C-terminus in an acid form. Cyclic peptides were obtained with a purity of approximately 90% by RP-HPLC, which is adequate for immunological studies. The identity of each peptide was determined by mass spectrometry. The molecular mass determined experimentally agreed with the theoretical mass calculated for each peptide (data not shown). The cyclic peptides were coupled to the BSA protein carrier through their C-terminus and the conjugation reactions were verified by immunoblotting analysis using specific antibodies for each peptide (Figure 1); OMV from the bacterium and BSA were used as positive and negative controls, respectively. Positive signals were obtained in the lane corresponding to the conjugates and OMV from *N. meningitidis*, while no signal

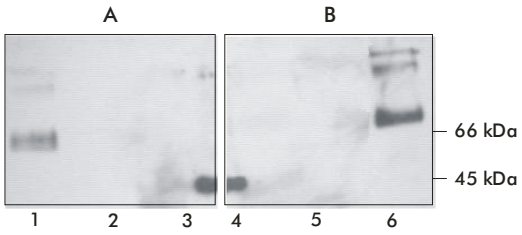

Figure 1. Immunoblotting analysis of BSA-conjugated PorA loop 1 (VR1) and 4 (VR2) peptides. Membranes were incubated with monoclonal antibodies that are specific to meningococcal PorA protein variable regions: A) loop 1 peptide (VR1, subtype P1.19) and B) loop 4 peptide (VR2, subtype P1.15). Lane 1) BSA-conjugated loop 1 peptide, lanes 2 and 5) BSA, lanes 3 and 4) outer membrane vesicles from *N. meningitidis* strain CU385, and lane 6) BSA-conjugated loop 4 peptide.

was observed in the lanes corresponding to the BSA protein.

After the immunization of BalB/C mice with the unconjugated peptide and the BSA-conjugated VR1 and VR2 peptides, sera were evaluated by ELISA against either the homologous peptides or OMV from *N. meningitidis*. Results are shown in figures 2 and 3, respectively. The antibody levels against all peptides were negligible after three doses of the antigen for the animals immunized with the carrier protein alone (data not shown). The unconjugated VR1 peptide was more immunogenic than the unconjugated VR2 peptide ($P < 0.001$). The immunogenicity of the VR2 peptide was increased after its conjugation to the protein carrier ($P < 0.05$). The levels of antibodies detected against the VR1 peptide were lower in sera form mice immunized with the BSA-conjugated VR1 peptide than those detected after immunization with the unconjugated VR1 peptide ($P < 0.001$).

Figure 3 shows that antibodies that specifically recognized the outer membrane of *N. meningitidis* were detected in all groups immunized with the unconjuga-

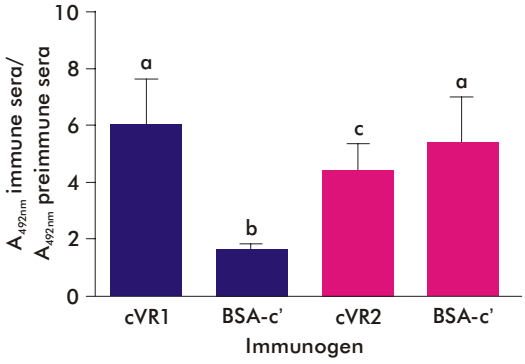

Figure 2. Anti-peptide antibody levels, measured by ELISA, of murine sera elicited against the unconjugated and the BSA-conjugated PorA VR1 and VR2 cyclic peptides. Plates were coated with the homologous unconjugated peptide. The results are expressed as the increases in absorbance signals at 492nm (A_{492nm}) of the third dose sera compared to the preimmune sera, after 10 min of incubation with the substrate. Sera were diluted 1/200. Legend: cVR1: cyclic VR1 peptide, BSA-cVR1: BSA-conjugated cyclic VR1 peptide, cVR2: cyclic VR2 peptide, BSA-cVR2: BSA-conjugated cyclic VR2 peptide. Bars represent the means and error bars represent the standard deviations for each group. Statistically significant differences are indicated with different letters.

17. Mocca LF, Frasch CE. Sodium dodecyl sulfate-polyacrylamide gel typing system for characterization of *Neisseria meningitidis* isolates. J Clin Microbiol 1982;16: 240-4.

18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-75.

19. Merrifield RB. Solid phase synthesis. Science 1986;232:341-7.

20. Houghten RA, Bray MK, Degraw ST, Kirby CJ. Simplified procedure for carrying out simultaneous multiple hydrogen fluoride cleavages of protected peptide resins. Int J Pept Protein Res 1986;27:673-8.

21. Andreu D, Albericio F, Sole NA, Munson NC, Ferrer M, Barany G. Peptide Synthesis Protocols. Totowa, New Jersey: Humana Press Inc., 1994.

22. Colowick SP, Kaplan NO. Methods Enzymol. 1983.

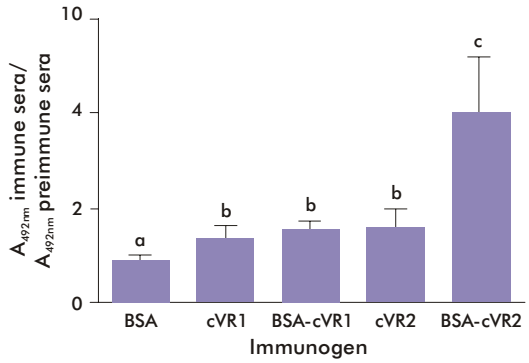

Figure 3. ELISA reactivity of murine sera, elicited against the unconjugated and the BSA-conjugated PorA VR1 and 2 cyclic peptides, with the outer membrane vesicle extracted from the meningococcal strain CU385. The results are expressed as increases in absorbance signals at 492nm (A_{492nm}) of the third dose sera compared to the preimmune sera, after 10 min of incubation with the substrate. Sera were diluted 1/200. Legend: cVR1: cyclic VR1 peptide, BSA-cVR1: BSA-conjugated cyclic VR1 peptide, cVR2: cyclic VR2 peptide, BSA-cVR2: BSA-conjugated cyclic VR2 peptide. Bars represent the means and error bars represent the standard deviations for each group. Statistically significant differences are indicated with different letters.

ted peptide and BSA-conjugated peptides: statistically significant differences in levels of antibodies directed against the membranes were detected in all groups immunized with peptides with regard to the control group immunized with BSA ($P< 0.001$ for the BSA-cVR2 group and $P < 0.05$ for the remaining groups). Similar levels of anti-OMV antibodies were detected in the groups immunized with the unconjugated peptide and BSA-conjugated VR1 peptide ($P > 0.05$). No statistically significant differences were found in the anti-OMV antibody titers detected for the groups immunized with the unconjugated VR1 and VR2 peptides ($P > 0.05$).

The levels of specific anti-peptide antibodies detected in sera of mice immunized with the BSA-conjugated loop 4 peptide were higher than those detected in the sera of mice immunized with the loop 4 unconjugated peptide, in agreement with previous reports [9, 11, 12], where high levels of anti-peptide antibodies were obtained against Por-A derived peptides after conjugation with protein carriers. These results concerning the immunogenicity and capacity of the elicited antibodies to recognize the meningococcal membrane obtained for the VR2 peptide subtype P1.15, are in accordance with our previous results [10-12]: we have found that polymers of the cyclic peptide [10], conjugate to the P64K protein of linear and cyclic peptides [11, 30] and the peptide displayed on the surface of filamentous phage M13 [12] were able to elicit specific anti-peptide antibodies. Moreover, the antibodies elicited were able to recognize the native PorA protein on the surface of the bacterium or even to elicit functional activity against the meningococcus [10, 12].

Certain modifications were made in the present study compared to our previous work with the VR1 peptide [11]. First, in the VR1 synthesized peptide we included all the amino acids of the loop 1 sequence. Second, we used the carbodiimide method for the conjugation reaction, which couples the peptide to the protein carrier specifically through the carboxylic groups of the peptide. In the specific case of the loop 1 peptide, there are only two possibilities for reaction: the γ-carboxylic group of glutamic acid and the COOH terminal group. Previously [11] the conjugation process was less specific due to the use of the glutaraldehyde method, that couples the protein to the amine groups of the peptide and the peptide fragment, derived from the VR1, that had four amine groups: three lysine and the NH_2 terminal group.

Here, in contrast to the previous report [11], we found that the unconjugated synthetic peptide corresponding to VR1 elicited higher antibody titers than the unconjugated VR2 peptide and the elicited antibodies were able to react with the *N. meningitidis* outer membranes. Hence, the differences in the anti-VR1 immune response found between these two studies are attributable to the differences in the peptide length assayed. These differences in length could lead to the differences detected in the amount and quality of the antibodies generated, either by including new B and T epitopes in the longer peptide, in addition to those present in the peptide that contained only the apex of the loop 1, or due to conformational differences between these two peptides.

Previous studies with animal and human sera showed that in the P1.15 subtype peptide conformation does not seem to be as important for antigenicity and immunogenicity [11] as in the P1.19 subtype. The importance of conformation of the PorA VR1 for the human immune response against *N. meningitidis* was demonstrated in a study where a cyclic peptide with the sequence of the complete VR1 (subtype P1.19) was better recognized by sera from individuals vaccinated with the Cuban anti-meningococcal BC OMV-based vaccine VA-Mengoc-BC® than a linear peptide with an identical sequence [paper pending publication]. This could be generally associated to the PorA VR1: Idänpään-Heikkilä and coworkers [2], found that the detection by immunoblotting of the immune response against loop 1 in the sera of individuals that were convalescing from meningococcal disease was enhanced in the presence of cluck detergents, while no differences were found in the immune response against loop 4 with or without the inclusion of detergents.

Therefore, we considered here that the main reason for the results obtained was the possible conformational change introduced by the increase in the peptide length. Evidence for length-dependent conformational epitopes has been previously reported [31]. Nevertheless, we do not absolutely reject the possibility that the introduction of new B and/or T-cell epitopes in the longest peptide, helped increase peptide immunogenicity. We do not consider this, however the basic reason, because B-cell epitopes of PorA have been mapped at the apex of the exposed loops [4, 32]. On the other hand, significant changes in immunogenicity due to the possible inclusion of new T-cells epitopes must be taken into account if they included transmembrane regions of the protein, because that is where the immunodominant T cell epitopes of PorA protein [33] are found. According to the reported T-cell epitope map of PorA, there are no significant differences in the presence of T-cell epitopes for peptides containing only the apex of loop 1 or the complete loop 1 [34].

23. Carter MJ. Techniques for conjugation of synthetic peptides to carrier molecules. In: Pennington MW, Dunn BM, editors. Peptide analysis protocols.Totowa NJ, Humana Press, 1994: p. 155-91.

24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227: 680-5.

25. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350-4.

26. Cruz S, Fernández de Cossío ME, del Valle J, Nazabal C, Ohlin M, Gavilondo JV. Monoclonal antibodies against P1, P3 and 31kDa outer membrane proteins of *Neisseria meningitidis* B:4:P1.15. Biotecnol Apl 1993;10:89.

27. van der Voort ER, van der LP, van der BJ, George S, Tunnela O, van Dijken H, *et al*. Specificity of human bactericidal antibodies against PorA P1.7,16 induced with a hexavalent meningococcal outer membrane vesicle vaccine. Infect Immun 1996;64:2745-51.

28. Martin DR, Ruijne N, McCallum L, O'hallahan J, Oster P. The VR2 epitope on the PorA P1.7-2,4 protein is the major target for the immune response elicited by the strain-specific group B meningococcal vaccine MeNZB. Clin Vaccine Immunol 2006;13:486-91.

29. Rosenqvist E, Hoiby EA, Wedege E, Bryn K, Kolberg J, Klem A, *et al*. Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. Infect Immun 1995;63:4642-52.

30. González S, Alvarez A, Caballero E, Vina L, Guillén G, Silva R. P64k meningococcal protein as immunological carrier for weak immunogens. Scand J Immunol 2000;52:113-6.

31. Pincus SH, Smith MJ, Jennings HJ, Burritt JB, Glee PM. Peptides that mimic the group B streptococcal type III capsular polysaccharide antigen 790. J Immunol 1998;160:293-8.

32. McGuinness BT, Lambden PR, Heckels JE. Class 1 outer membrane protein of *Neisseria meningitidis*: epitope analysis of the antigenic diversity between strains, implications for subtype definition and molecular epidemiology. Mol Microbiol 1993;7:505-14.

33. Wiertz E, van Gaans-van den Brink, Hoogerhout P, Poolman J. Microheterogeneity in the recognition of a HLA-DR2-restricted T cell epitope from a meningococcal outer membrane protein. Eur J Immunol 1993;23:232-9.

34. Wiertz EJ, van Gaans-van den Brink JA, Gausepohl H, Prochnicka-Chalufour A, Hoogerhout P, Poolman JT. Identification of T cell epitopes occurring in a meningococcal class 1 outer membrane protein using overlapping peptides assembled with simultaneous multiple peptide synthesis. J Exp Med 1992;176:79-88.

Surprisingly, the BSA-conjugated loop 1 peptide elicited lower levels of antibodies against the pepti-de than the unconjugated peptide (Figure 2), but the analysis of the *N. meningitidis* outer membrane recognized by ELISA showed that similar levels of antibodies that are able to react with the meningococcal membranes were detected in the sera of animals immunized with both immunogens. It implies that the unconjugated VR1 peptide, even though it is immunogenic, induced certain antibodies against the unfolded conformation, that were weakly reactive or failed to react with the native protein. Synthetic peptides are linear arrays of amino acids that mainly have a random structure when in solution [21]. While it is not difficult to produce antipeptide antibodies, it does not necessarily follow that the antibodies will recognize a protein containing the same stretch of the sequence found in the peptide [35]. Also, the epitopes recognized by B cells include mobile peptides containing sequential or non-sequential amino acids [36]. We therefore speculate that after immunization with the unconjugated peptide a pronounced immune response was induced against some peptide conformations that are different from the same sequence in the protein, and thus it is not totally recognized. In the case of the conjugated VR1 peptide, the conjugation process probably limited the number of possible peptide conformations, including the possible immunodomi-nant epitopes formed in the unconjugated peptide, reducing the levels of antibodies against the peptide, but these limited numbers of conformations remaining seems more similar to the natural ones, which could explain their capacity to bind to the meningococcal membranes.

In summary we report here that antibodies against synthetic peptides with sequences corresponding to the meningococcal PorA subtype P1.19 are able to recognize the native PorA protein in the outer membrane of the bacterium, and that the epitope involved in this recognition must be presented to the immune system in a way that is as close as possible to the natural one to obtain an adequate immune response.

Acknowledgments

The authors are very grateful to colleagues from the Finlay Institute for providing us with the meningococcal strain CU385 used in this work.

35. Rothbard JB. Synthetic peptides as vaccines. Biotechnology 1992;20:451-65.

36. Kuby J. Immunology. 3rd ed. New York: WH Freeman and Company, 1997.

Received in August, 2008. Accepted for publication in December, 2008.