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Anti-PorA Antibodies Elicited by Immunization with Peptides Conjugated to P64k

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To increase the humoral immune response against two cyclic synthetic peptides, derived from variable regions within the outer membrane meningococcal protein PorA (subtypes 19 and 15), we conjugated the peptides to P64k, a novel carrier protein from the same bacterium expressed in *Escherichia coli*. In addition, one of these peptides was restricted to a linear conformation before it was chemically coupled to the carrier. The conjugates were administered to mice in a three-dose immunization schedule, resulting in a potent anti-peptide immune response, which suggested that chemical conjugation to this carrier provided T-cell help. Antisera directed to the three conjugates reacted with *Neisseria meningitidis* outer membrane PorA upon immunoblot analysis. Moreover, in two out of three conjugates, the anti-peptide sera reacted with native meningococcal outer membrane vesicles in ELISA.

Key Words: **PorA; P64k; meningococci; cyclic peptide; conjugated peptide; carrier protein.**

Peptide vaccines can provide an effective vaccine by focusing the host's immune response on epitopes known to play a role in protective immunity (1). Among the major outer membrane proteins of *Neisseria meningitidis*, the porin PorA has been considered as an important vaccine candidate. Murine antibodies against it have been effective in generating a protective response in an infant rat infection model (2). Moreover, a correlation between the bactericidal activity of human immune sera and the levels of PorA-specific antibody titers has been reported (3). A two-dimensional model of PorA protein predicts the presence of eight cell surface-exposed loops in the protein (4). The sequence variation in the genes (*porA*) encoding this antigen in a number of reference strains was found to be largely confined to two regions designated VR1 and VR2, located on loop 1 and loop 4, respectively (5). Having into account that certain epitopes on its variable regions seem to be the target of protective antibodies, synthetic peptides derived from the sequence of PorA protein (subtypes 16a and 16b) have been assayed as candidate antigens (6, 7).

CU 385 represents the main wave of strains responsible for meningococcal meningitis in Cuba in the last 15 years. In a previous work, two cyclic peptides, derived from VR1 (Garay, H. E., unpublished result) and VR2 (8) of the PorA protein expressed by this strain, were synthesized and tested as immunogens in Balb/c mice, eliciting antibodies with a moderated functional activity. In the present study, we conjugated these peptides to P64k, a novel carrier protein from the same bacterium expressed in *Escherichia coli* (9), and investigated the immunogenicity of the conjugates in mice.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Neisseria meningitidis* strain CU385 (B:4:P1.19,15) has been described previously (10). The strain was grown on brain–heart infusion agar at 37°C for 18 h in an atmosphere of 5% (v/v) CO_2.

Outer membrane vesicles. Outer membrane vesicles (OMV) were prepared by extraction of whole meningococci with lithium acetate as described elsewhere (11).

Synthetic peptides. The following peptides derived from the surface loop 1 (variable region 1) and loop 4 (variable region 2) of PorA outer membrane protein from the *Neisseria meningitidis* strain CU385 were used in this study: VR1, cyclic-CNFQLQLTEPPSKSQPQVKVTKC; VR2, cyclic-CPIQNSKSAYTPAHYTRQNNADVFVPAVVGKPGSC; $VR2_L$, S-carboxymethylated-CPIQNSKSAYTPAHYTRQNNADVFVPAVVGKPGSC.

Solid-phase peptide synthesis was carried out manually using the Boc/Bzl chemistry as described formerly (8). The reduced peptides were cyclized with 20% dimethyl sulfoxide in water (12). The S-carboxymethylation was accomplished as reported previously (13). The cyclic and S-carboxymethylated peptides were purified by reversed-phase HPLC and characterized by mass spectrometry.

Conjugation and conjugate characterization. Recombinant P64k protein was obtained as described earlier (9). The peptides were conjugated to this carrier protein by the glutaraldehyde method, as previously described (14). After coupling, protein concentration was

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determined using Lowry's method (15) and peptide–protein conjugates were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (16). Additionally, the conjugates were analyzed by Immunoblot (17). The nitrocellulose membranes were probed with either a PorA subtype 19- or a PorA subtype 15-specific monoclonal antibody (Mab), depending on the peptide (18; Nazábal, C., unpublished result).

Immunizations. Female Balb/c mice (H-2^d) of 8–10 weeks of age were immunized. Ten micrograms of either conjugate, free peptide or P64k were diluted in phosphate-buffered saline (PBS), emulsified with Complete Freund's Adjuvant, and subcutaneously (s.c.) administered to mice (n = 8) divided in seven experimental groups. Animals received two more doses of conjugate, carrier or peptide, emulsified in Incomplete Freund's Adjuvant at 2-week intervals. Serum samples were obtained from mice at days 0, 14, 28, and 42.

ELISA. Antibody levels in sera were determined by Enzyme Linked Immunosorbent Assay (ELISA). To detect anti-peptide antibodies, 96-well plates (High Binding, Costar, USA) were coated with 100 μl/well of corresponding peptide (20 μg/ml) in carbonate buffer (0.05 M Na_2CO_3, pH 9.6). Bovine serum albumin (2%) was used as a blocking reagent. Plates were processed as published elsewhere (19). All sera were analyzed in duplicate. Serum anti-peptide antibody levels were expressed as their absorbance (492 nm) values in ELISA and used for statistical analysis. The presence of anti-native PorA antibodies in sera was determined by a similar procedure, using plates coated with 10 μg/ml of OMV prepared from strain CU385.

Analysis of anti-peptide sera by immunoblot. Meningococcal OMV (10 μg/lane) were separated by SDS–PAGE and transferred to a nitrocellulose membrane. After blocking with skim milk powder (5%) in PBS, the lanes were separated and incubated with either carrier-specific antisera or antisera elicited against conjugated peptide (pooled antisera diluted 1/500). This procedure was followed as previously described (17).

Statistical methods. Statistically significant differences for anti-peptide antibody levels were assessed by Kruskal–Wallis test, followed by Dunne's Multiple Comparison Test. The significance of differences in anti-native PorA antibody levels was determined by ANOVA and Newman–Keuls Multiple Comparison Test. A P value of <0.05 was considered statistically significant. In the figures, bars represent the mean of antibody levels ± the standard deviation for each experimental group.

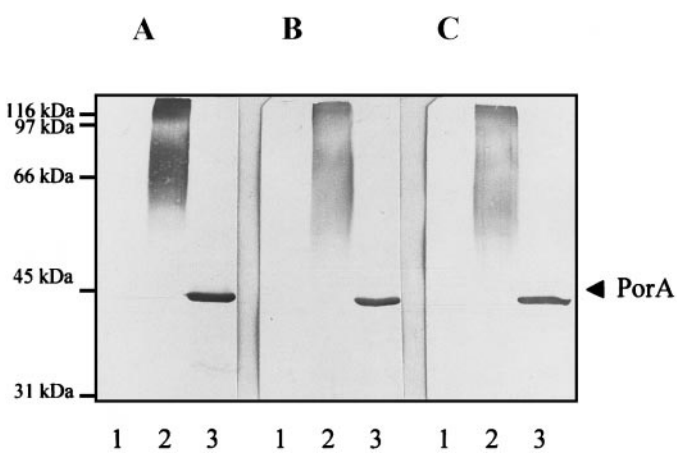

FIG. 1. Immunoblots showing recognition of meningococcal PorA-derived peptides conjugated to P64k by PorA-specific antibodies. (A) VR1 peptide; (B) VR2 peptide; (C) $VR2_L$ peptide. In each panel: lane 1, P64k (2 μg); lane 2, peptide conjugated to P64k (3 μg); lane 3, OMV from meningococcal strain CU385 (10 μg).

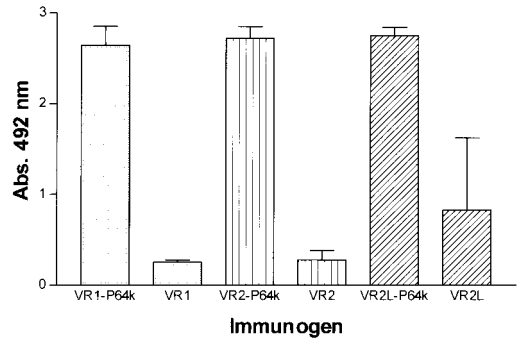

FIG. 2. Anti-peptide antibody levels. Mice (n = 8) were s.c. immunized with three doses (10 μg/dose) of free VR1, VR2, $VR2_L$ or the same amount of the respective P64k conjugate. Antibody levels in sera were measured against the homologous peptide and are expressed as their absorbance (492 nm) values in ELISA (serum dilution 1:100).

RESULTS AND DISCUSSION

Previously, cyclic peptides corresponding to VR1 and VR2 regions of the meningococcal PorA protein have been designed and employed as candidate antigens with good results (7, 20). These reports reinforced the necessity of mimicking the PorA protein surface loop when designing new synthetic immunogens. Accordingly, two cyclic peptides derived from the loop 1 (Garay, H. E., unpublished result) and loop 4 (8) sequences of the meningococcal strain CU385 were designed and assayed as antigens in mice. Both peptides produced a humoral immune response when they were administered to Balb/c mice, but the functional activity of the antibodies was moderated.

In the present work, we linked such peptides to P64k. In addition, for comparison, one of them was *S*-carboxymethylated and conjugated to the same carrier in a parallel coupling reaction. Figure 1 shows the recognition of the resulting conjugates by either P1.19- or P1.15-specific Mabs in immunoblot. This procedure indicated similarity in the electrophoretic pattern of the three conjugates.

P64k, the conjugates and the uncoupled peptides were administered to mice in a three-dose schedule. Figure 2 resumes the anti-peptide antibody levels found in murine sera, after three doses of coupled and uncoupled peptide. Each serum was tested in duplicate in ELISA, using the immunizing peptide as the coating antigen. As it can be seen, the immunogenicity of the peptides was greatly increased after their conjugation to P64k. In all cases, there was statistically significant difference between the group immunized with conjugate and the group that received free homologous peptide. When the anti-conjugated peptide antisera were titrated in ELISA by serial twofold dilution we found that anti-peptide titers were higher than 25,600 (data not shown). The antibody levels against all peptides

remained negligible, even after three doses of antigen, for the animals immunized with the carrier protein alone (data not shown).

The increased immunogenicity observed for the conjugated peptides is in agreement with previous reports (7, 20), where high levels of anti-peptide antibodies were obtained against PorA-derived peptides conjugated to traditional protein carriers, like tetanus toxoid and keyhole limpet hemocyanin. However, we have coupled these peptides to the P64k protein, a meningococcal antigen expressed in *E. coli* by recombinant DNA technology. We have recently reported that P64k is a suitable protein carrier for weak immunogens (21). The P64k-containing conjugates could prime the host for a specific meningococcal T-cell memory response, by employing a carrier protein derived from the same bacterium.

The specificity of reactivity of all anti-conjugated peptide antisera was determined by Immunoblot against OMV extracted from strain CU385. As it can be seen in Fig. 3, all the conjugates elicited a PorA-specific response upon immunization. As expected, the P64k-specific antisera (lane 1) did not recognize the porin present in meningococcal membranes and only reacted with a weak band corresponding to the homologous protein present in meningococci.

Polyclonal antisera raised against synthetic peptides may contain antibodies directed to several conformations of the peptide, most of them unfolded, weakly reacting or failing to react with the native protein (22). Figure 4 shows the extent of reactivity of all antisera with intact meningococcal outer membranes in ELISA. As coating antigen, OMV prepared from strain CU385 were employed. It can be observed that antisera produced against VR1-P64k reacted with OMV, in ELISA, in a similar magnitude to antisera elicited against the carrier protein P64k. It indicates that this conjugate induced antibodies that predominantly recognized the PorA protein in an unfolded conformation, as antibodies against it did recognized the membranes in Immunoblot.

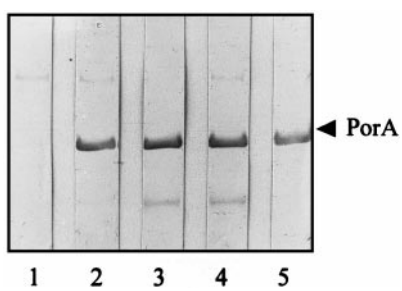

FIG. 3. Immunoblots of antisera obtained by immunization with synthetic peptides conjugated to P64k. Sera were raised against P64k (lane 1), VR1-P64k (lane 2), VR2-P64k (lane 3) and $VR2_L$-P64k (lane 4) and were tested against OMV extracted from strain CU385. Lane 5, murine monoclonal antibody specific to meningococcal PorA (subtype 15), as a positive control.

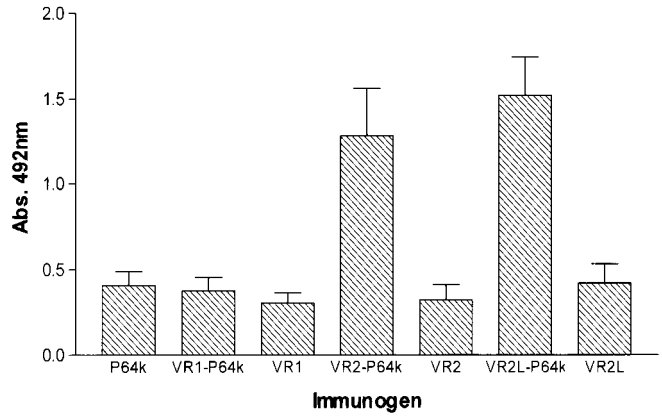

FIG. 4. Reactivity with OMV exhibited by antisera elicited against conjugated peptides. Anti-native PorA antibody levels in sera are expressed as their absorbance (492 nm) values in ELISA (serum dilution 1:100). The plates were coated with OMV (10 μg/ml) extracted from the meningococcal strain CU385.

Both conjugates prepared from peptides derived from loop 4 (VR2 and $VR2_L$) induced anti-native PorA antibodies. There was no statistically significant difference in the anti-PorA protein antibody levels elicited by both conjugated peptides. It suggests that both antisera contain antibodies directed to conformational epitopes within the PorA protein. Our results are encouraging, considering that previous studies revealed that even when almost all PorA-derived conjugated peptides evoked an anti-peptide titer, only a few were able to raise a significant anti-native PorA antibody response (7).

The difference found by us in the ability of loop 1- and loop 4-derived peptides to induce an anti-native protein antibody response might be attributable to the peptide length. VR1 peptide only comprises the apex amino acids of loop 1, whereas VR2 and $VR2_L$ include the full sequence of loop 4.

In summary, we report here that P64k can be an efficient carrier protein for PorA-derived peptides (in either cyclic or linear conformation). Chemical conjugation to this carrier granted T-cell help to these synthetic peptides, did not affect their folding and allowed them to induce a PorA-specific immune response.

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