INFECTION AND IMMUNITY, Jan. 2008, p. 334–338 Vol. 76, No. 1
0019-9567/08/$08.00+0 doi:10.1128/IAI.00943-07

A DNA Fusion Vaccine Induces Bactericidal Antibodies to a Peptide Epitope from the PorA Porin of *Neisseria meningitidis*[▽]

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Received 11 July 2007/Returned for modification 15 August 2007/Accepted 15 October 2007

An experimental DNA plasmid vaccine was developed based on a well-characterized and protective peptide epitope derived from a bacterial porin protein. For this study, we used the P1.16b serosubtype epitope, located in variable region (VR)2 in loop 4 of the PorA outer membrane (OM) porin from *Neisseria meningitidis* serogroup B strain MC58. A plasmid that encoded the entire loop (pPorAloop4) was prepared, as well as a fusion plasmid that encoded the loop in tandem with the fragment C (FrC) immunostimulatory sequence from tetanus toxin (pPorAloop4-FrC). The constructs were used for intramuscular immunization without exogenous adjuvant. Murine antisera raised to the pPorAloop4-FrC DNA fusion plasmid reacted significantly with OMs in enzyme-linked immunosorbent assay and with whole bacteria by immunofluorescence, whereas antisera raised to the pPorAloop4 DNA plasmid and to control plasmid showed little or no reactivity. Significantly, only the pPorALoop4-FrC plasmid induced bactericidal antibodies, demonstrating that the intrinsic immunostimulatory sequence was essential for inducing a protective immune response. The antibodies raised to the P1.16b pPorALoop4-FrC plasmid were serosubtype specific, showing no significant immunofluorescence reactivity or bactericidal activity against other PorA variants. These data provide proof of principle for a DNA fusion plasmid strategy as a novel approach to preparing vaccines based on defined, protective epitopes.

DNA vaccines have been the focus of intense investigation over the past two decades (12, 23). Essentially, they consist of bacterial plasmid DNA into which genes encoding antigens are placed, with gene expression commonly driven by a strong viral promoter. Delivery into muscle or skin cells results in antigen production and presentation to the immune system, leading to both antibody and cell-mediated immune responses. DNA vaccines for therapies against autoimmune diseases, allergies, and cancers such as follicular lymphoma are in development (7, 33, 34). In addition, the ability of DNA vaccines to induce both humoral and cellular immune responses has been demonstrated in a number of human clinical trials and experimental models of infectious human diseases caused by viruses (4, 25, 39), intracellular bacteria (11, 36), and parasites (20, 32, 38). The potential of DNA vaccination in domestic livestock and pet animals has also been explored (8, 9, 13, 22), and several vaccines have now been licensed for veterinary use (2, 3).

DNA vaccines have been reported to induce antibody responses against bacterial pathogens where humoral immunity to protein antigens is believed to be essential, e.g., against *Borrelia burgdorferi* outer surface proteins (37), *Bacillus anthracis* soluble LF toxin (30), outer membrane (OM) porin OprF of *Pseudomonas aeruginosa* (29), and PorB protein of *Neisseria gonorrhoeae* (44). For the last, although antibodies were induced in mice, they were not bactericidal for gonococci, thus identifying that both the native conformation of antigen and antibodies of high titer and avidity are prerequisites for generating protective immune responses.

The experience with the gonococcal porin suggests that the DNA vaccine approach may not be suitable for whole bacterial proteins that adopt complex conformations in the OM. In the current study, a strategy was developed to investigate whether it was possible to focus the humoral antibody response towards a defined bacterial porin epitope that is known to be essential for inducing functional, bactericidal antibodies (6). To provide proof of principle of this peptide epitope-based DNA vaccine approach, we used the well-characterized protective epitope from the P1.7,16b serosubtype PorA OM porin from *Neisseria meningitidis* serogroup B strain MC58. Within the meningococcal OM, this protein is organized as a series of conserved regions forming amphipathic transmembrane β-sheets that generate eight surface-exposed loops (35). The protective P1.16b epitope is conformational and located in the variable region (VR)2 at the apex of loop 4, which is the longest (36 amino acids) and most accessible to immune recognition (26, 27, 28). Data are presented that demonstrate the potential of an experimental DNA plasmid vaccine containing the P1.16b epitope to induce a protective, bactericidal immune response against serogroup B meningococci.

MATERIALS AND METHODS

Bacteria and growth conditions. *Neisseria meningitidis* strain MC58 (B:15:P1.7,16b) was isolated from an outbreak of meningococcal infections that occurred in Stroud, Gloucestershire, United Kingdom, in the mid-1980s (27), and

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▽ Published ahead of print on 29 October 2007.

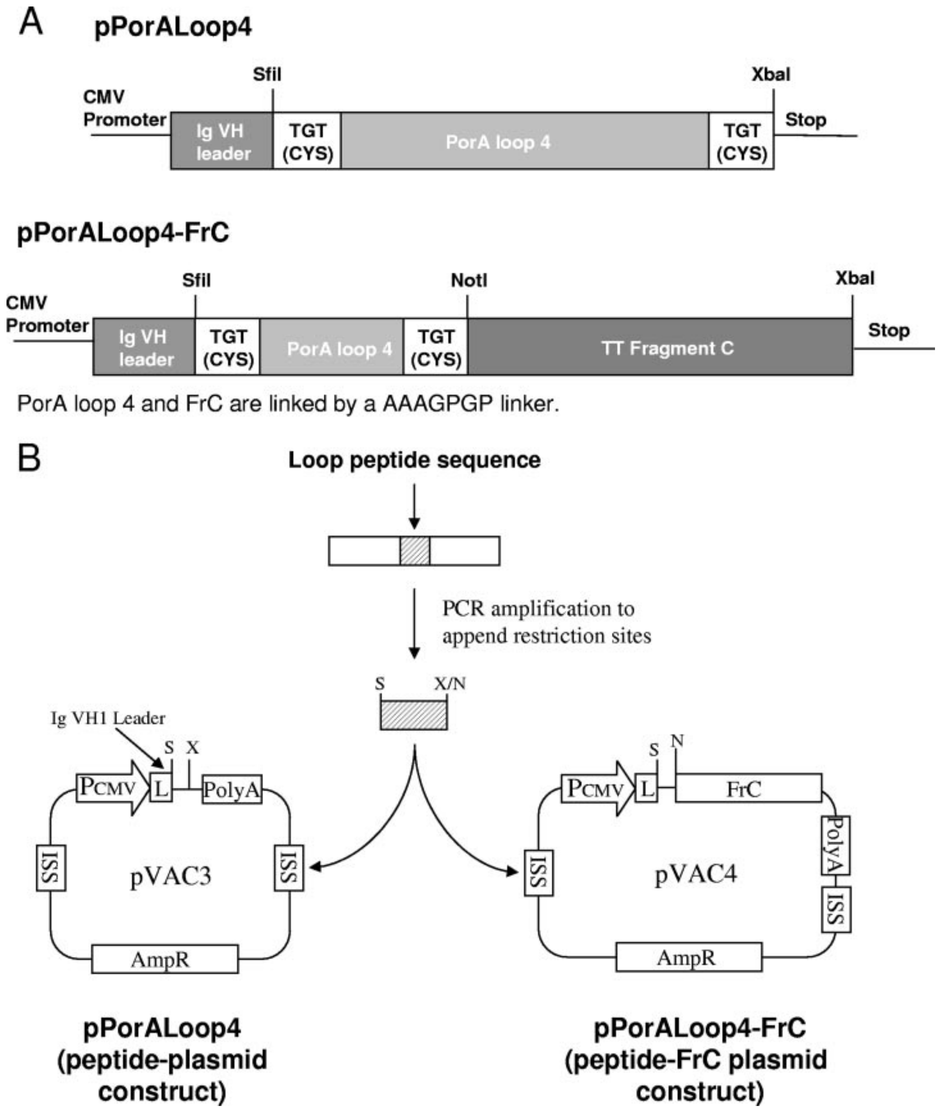

FIG. 1. (A) Structures of pPorALoop4 and pPorAloop4 conjugated to FrC, showing positions of cysteines. (B) Schematic of the preparation of pPorALoop4 peptide-plasmid and pPorALoop4-FrC plasmid vaccine constructs. P_{CMV}, cytomegalovirus early promoter; PolyA, polyadenylation signal sequence from bovine growth hormone; ISS, immunostimulating sequence; AmpR, ampicillin resistance gene; N, NotI; S, SfiI; X, XbaI.

Neisseria meningitidis strain H44/76 (B:15:P1.7,16) is the subtype P1.7,16 reference strain (10). *Neisseria meningitidis* strains MC50 (C:NT:P1.21,16), MC106 (C:4:P1.7,9), and MC168 (B:4:P1.5,2) have been described previously (17, 28).

Bacteria were grown on supplemented proteose-peptone agar (43) incubated at 37°C in an atmosphere containing 5% (vol/vol) CO_2. OMs were prepared by extraction of wild-type MC58 whole cells with lithium acetate as described previously (14). OM vesicles (OMV) were produced by extraction of the OM with sodium deoxycholate according to the protocol described by Christodoulides et al. (5).

Construction of peptide epitope-based DNA plasmid vaccines. DNA vaccine constructs were prepared that encoded the entire surface-exposed loop 4 (36 amino acids) containing the protective VR2 P1.16b epitope of the PorA protein (pPorALoop4), with and without the presence of the fragment C (FrC) immunostimulatory sequence from tetanus toxin. In order to construct the pPorALoop4-FrC DNA plasmid vaccine, partially complementary sense and antisense oligonucleotides (PorALoop4 primer 1 [5′-TATAGGCCCAGCCGGCCATGGCCTGTCCCATCCAGAACAGCAAGTCCGCCTATACCCCAGCTTACTACACCAAGAACACC-3′] and PorALoop4 primer 2 [5′-TATAGCGGCCGCGCAGGATCCGGGCTTGCCGACCACGGCAGGCACGAGAGTCAGATTATTGTTGGTGTTCTTGGTGTAGTAAGC-3′]) were annealed and amplified by PCR to obtain a human codon optimized PorA loop 4 DNA fragment. Additional cysteine codons were included at both the N and C termini in order to provide the potential conformational constraint of a disulfide bridge, mimicking the approach previously used with synthetic peptides (6). This fragment was reamplified (with PorALoop4XbaR primer [5′-TATATCTAGACTAGCAGGATCCGGGCTTG-3′]) to include a stop codon and an XbaI site at the 3′ end (Fig. 1A). The pPorALoop4 DNA fragment, without a FrC sequence, was also similarly prepared. The fragments were then digested with SfiI and NotI restriction enzymes and inserted into pcDNA3-based vectors (pVAC3 and pVAC4) that contained a built-in leader sequence derived from a human immunoglobulin V_H gene, to produce a pPorALoop4 DNA plasmid vaccine and a pPorALoop4-FrC DNA plasmid vaccine, respectively (Fig. 1B). Control DNA plasmid pFrC, containing the FrC fragment without the pPorALoop4 DNA fragment, was also prepared.

Immunization of animals. Groups of five BALB/c (H-2^d haplotype) and C57BL/6 (H-2^b haplotype) mice at 6 to 7 weeks of age of approximately equal weight were used for immunization. Each animal was immunized intramuscularly with 50 μg of pPorAloop4 plasmid or pPorAloop4-FrC plasmid in saline (0.9% [wt/vol] NaCl) without adjuvant at days 0, 21, and 42. The dose was split between both hind legs, and animals were sacrificed 14 days after the final immunization. Groups of control mice (n = 5) were injected with control DNA plasmid or saline alone. In addition, groups of BALB/c mice (n = 5) were immunized subcutaneously on days 0 and 21 with 10 μg per animal of either MC58 OM or OMV in saline. All sera were stored at −20°C. This study complied with the animal experimentation guidelines of the authors' institution.

Detection of the immune response. (i) ELISA. Individual murine antisera were reacted in enzyme-linked immunosorbent assay (ELISA) against OM from strain MC58 as described previously (6). Absorbance was measured at 450 nm after 10 min of incubation with enzyme substrate, and geometric mean (GM) ELISA titers (± 95% confidence limits) were extrapolated from linear portions of titration curves and taken as the reciprocal dilution that gave an absorbance increase of 0.1 h^{-1} (6). A two-sample *t* test was used to compare the mean levels of absorbance between groups of mice immunized with different preparations, with a P value of <0.05 being significant.

(ii) Immunofluorescence. The reactivity of murine antisera with PorA present in the OM of meningococcal cells was detected by immunofluorescence as described previously (5). Briefly, pooled murine antisera (1/50 dilution) were reacted with methanol-fixed bacteria, and bound antibody was detected by reactivity with anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate (Dako, United Kingdom) (5). As a positive control, antiserum raised to OM of MC58 was used. The cells were then examined using a Leica model TCS 4D confocal microscope (Leitz), and images were constructed from 20 optical sections, obtained using the same level of fluorescein isothiocyanate excitation for each pooled antiserum sample.

(iii) Serum bactericidal assay. The bactericidal activity of pooled murine antisera was determined against the homologous strain MC58 and heterologous strains H44/76, MC50, MC106, and MC168 essentially as described by Christodoulides et al. (6), using 5%% (vol/vol) baby rabbit complement as an exogenous source of complement. Meningococci were harvested from solid agar plates into Dulbecco B phosphate-buffered saline (PBSB) containing 1% (vol/vol) heat-inactivated (56°C, 30 min) fetal calf serum. Bacterial suspension (25 μl, containing approximately 1,000 CFU) was added to the wells of a sterile 96-well microtiter plate containing serial dilutions of test antisera, which had been previously decomplemented, in PBSB (10 μl). Freshly thawed baby rabbit complement was added and the plates agitated briefly to ensure thorough mixing. The plates were incubated at 37°C for 30 min in an atmosphere of 5% (vol/vol) CO_2, and then 15-μl samples were removed from each well for determination of surviving CFU. All sera, with and without exogenous complement, were assayed in triplicate at each serial dilution. Samples were also taken from control wells containing meningococci in PBSB with exogenous complement but without test antiserum. The serum bactericidal activity was recorded as the highest dilution at which ≥50% killing of strain MC58 was observed, and values are the mean determinations of assays carried out in triplicate.

RESULTS AND DISCUSSION

DNA vaccine constructs encoding the entire surface-exposed loop 4 (36 amino acids) containing the protective VR2 P1.16b epitope of the PorA protein (pPorALoop4) were prepared (Fig. 1). To increase potency, a fusion design was used in which the peptide was linked with the FrC immunostimulatory sequence from tetanus toxin. The FrC fragment, which has been shown to increase antibody responses to cancer DNA vaccines (18), provides cognate T-helper function through the expression of "universal" $CD4^+$ T-cell epitopes contained within the sequence, facilitates peptide folding, and increases peptide stability. The immunogenicities of the pPorALoop4 DNA plasmid and pPorALoop4-FrC DNA plasmid constructs were investigated in mice, following intramuscular immunization without the use of exogenous adjuvant.

Antisera from BALB/c and C57BL/6 mice immunized with control DNA plasmid, pPorALoop4 DNA plasmid, or saline showed little or no reactivity (GM titers of ≤600; $P > 0.05$) against MC58 OM in ELISA (Fig. 2A). By contrast, both BALB/c and C57BL/6 mice immunized with the pPorALoop4-FrC DNA plasmid construct showed weak but significant ELISA reactivity [GM titers (95% confidence limits) of 1,400 (300, 6,800) and 1,100 (800, 1,600) for BALB/c and C57BL/6 mice, respectively] compared with any of the other treatments ($P < 0.05$) (Fig. 2A).

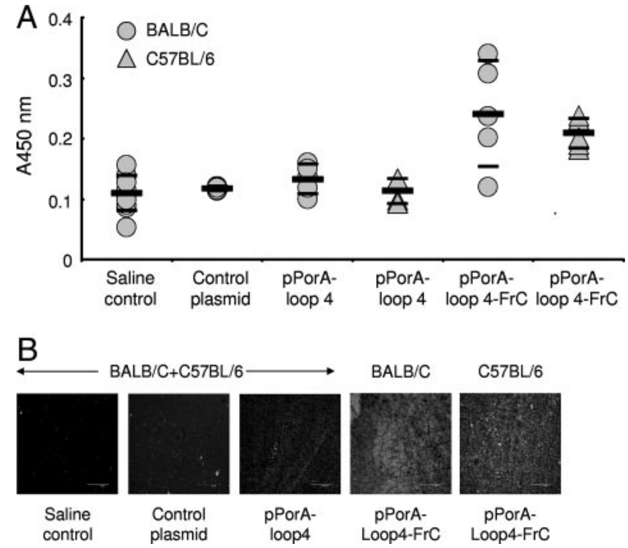

FIG. 2. (A) Reactivities of antisera from BALB/c and C57BL/6 mice in ELISA, measured against OM of *Neisseria meningitidis* MC58. Each point represents an individual serum, and the bars represent the mean and standard deviations for each group of animals. Reactivity is shown for a dilution of 1/100 of each antiserum, with A_{450} values obtained after 10 min. Results for C57BL/6 antisera raised to controls (not shown) were similar to those observed with BALB/c mice. (B) Pooled antisera from BALB/c and C57BL/6 mice immunized with the pPorALoop4-FrC plasmid construct react with whole MC58 bacteria as determined by immunofluorescence. Bars, 75 μm.

Next, the ability of antisera to react with whole bacteria was investigated by immunofluorescence, since positive reactivity in this assay has shown better correlation with serum bactericidal activity than reactivity in ELISA (5, 15, 16, 42). Pooled murine antisera from BALB/c mice immunized with the pPorALoop4-FrC DNA plasmid construct reacted with whole MC58 bacteria, whereas little or no reactivity was observed with antisera to the pPorALoop4 DNA plasmid or to control DNA plasmid or with serum from saline control animals (Fig. 2B). Similarly, only antisera raised to the pPorALoop4-FrC DNA plasmid construct in C57BL/6 mice showed positive immunofluorescence (Fig. 2B).

We next used immunofluorescence reactivity to determine whether antibodies raised to the pPorALoop4-FrC DNA plasmid construct were specific for the PorA VR2 P1.16b epitope. Positive immunofluorescence reactivity was observed against the homologous strain MC58 (P1.7,16b) (Fig. 3). However, no significant immunofluorescence reactivity was observed against meningococcal strain H44/76 (P1.7,16), MC50 (P1.21,16), MC106 (P1.7,9), or MC168 (P1.5,2) (Fig. 3). Therefore, the antibodies generated were specific for the P1.16b serosubtype and showed no cross-reactivity with strains expressing either the closely related P1.16 epitope or unrelated VR2 epitopes. Despite this serosubtype-specific response, the antisera raised to the pPorALoop4-FrC DNA plasmid showed no significant reactivity against the homologous MC58 OM in Western blotting (data not shown), suggesting that the expressed peptide

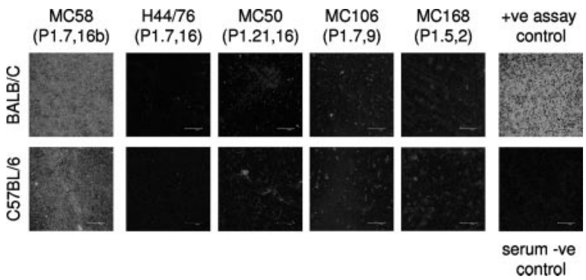

FIG. 3. Reactivities of antibodies raised to pPorALoop4-FrC against different meningococci as determined by immunofluorescence. Pooled antisera raised in BALB/c and C57BL/6 mice were reacted against strains MC58 (P1.7,16b), H44/76 (P1.7,16), MC50 (P1.21,16), MC106 (P1.7,9), and MC168 (P1.5,2). As a positive assay control, antiserum (1/100 dilution) raised to OM was reacted against the homologous strain MC58. Negative serum controls were tested for each strain for which a representative image for MC58 is shown. Bars, 75 μm.

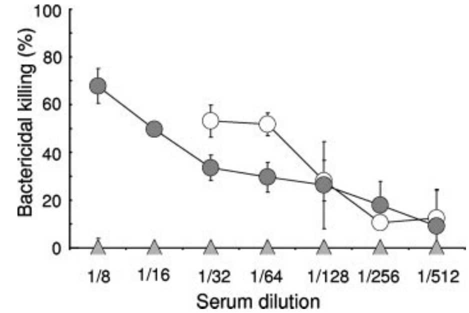

FIG. 4. Serum bactericidal activities of pooled antisera from BALB/c (●) and C57BL/6 (○) mice immunized with the pPorALoop4-FrC plasmid construct. Values are mean levels of killing and error bars the standard deviations from triplicate bactericidal killing experiments. Sera from mice of both haplotypes were nonbactericidal for strains H44/76, MC50, MC106, and MC168 (△).

elicited an antibody response that was likely to be directed towards a conformationally restricted epitope.

Next, the bactericidal activity of pooled murine antisera against the homologous strain, MC58, was determined. Significant bactericidal activity, i.e., 50% end point titers of 1/16 and 1/64 serum dilutions (Fig. 4), was demonstrated by pooled antisera raised to the pPorALoop4-FrC DNA plasmid construct in BALB/c and C57BL/6 mice, respectively. By comparison, the MC58 OM and OMV preparations induced serum bactericidal activity with 50% end point titers of approximately 1/6,400 and 1/800 serum dilutions, respectively. Antisera raised to control DNA plasmid alone, raised to pPorALoop4 plasmid, or from saline control animals, of either haplotype, were nonbactericidal (50% end point titers of <1/4). Notably, the bactericidal activity of antisera raised to pPorALoop4-FrC was serosubtype specific, as no killing of strain H44/76, MC50, MC106, or MC168 was observed (Fig. 4).

The construction of experimental antibacterial DNA vaccines that encode peptide sequences has been attempted previously, but this has focused on peptide mimetics of bacterial capsular polysaccharides (CPS) without attempting conformational constraint. Murine antibodies have been induced to a 15-amino-acid peptide mimetic of *Streptococcus pneumoniae* serotype 4 CPS, but no bactericidal activity was reported (21). In addition, a multiepitope DNA vaccine encoding a 73-amino-acid polypeptide which contained a peptide (13-amino-acid) mimetic of meningococcal serogroup C CPS in tandem with an adenoviral secretory leader sequence and two human immunodeficiency virus Th cell epitope sequences has also been reported to induce a significant antipolysaccharide antibody response that was bactericidal and protected mice from lethal meningococcal challenge (31). More recently, DNA vaccines encoding anti-idiotypes and peptide mimetics of the group B meningococcal capsule have also been reported to induce bactericidal antibodies in mice, which also protected infant rats from bacteremia (1, 24).

Our current study provides proof of the feasibility of the peptide epitope-based DNA vaccine strategy for inducing humoral antibodies to a defined, conformation-dependent epitope derived from a bacterial porin. However, the murine antibody titers and bactericidal responses were lower than those previously observed for rabbit antisera raised to an analogous 36-amino-acid synthetic cyclic peptide (a 50% end point titer of 1/320 rabbit serum dilution) (6), although those results were obtained using Freund's adjuvant, which is not suitable for human use. Moreover, bactericidal activity was weak compared with that of the murine antisera raised to OM and OMV prepared for this study. Therefore, even though the PorA loop 4 peptide sequence contains Th cell epitopes (41), it is likely that addition of an exogenous adjuvant would significantly increase immunogenicity (19). Furthermore, intramuscular injection may be suboptimal for delivery of peptide epitope-based DNA vaccines, and other methods that enhance immunogenicity, such as electroporation (40), could be used. It is also possible that the position of the conformational constraint introduced into the DNA sequence encoding the loop peptide was not ideal. However, the DNA vaccine technology more readily enables the production of many constructs in which the position of the cross-linking bridge(s) can be varied than does the more time-consuming and expensive chemical synthesis of peptides. The current study has also shown that the induced antibody response was serosubtype specific, and an important advantage of the peptide epitope-based DNA vaccine approach is that new DNA vaccines could be tailored to other variable epitopes. In the case of PorA, it can be envisaged that vaccines encoding other serosubtypes could be rapidly produced in response to any changes in the immunodominant epitope sequences occurring through immune selection within a given population.

In summary, the peptide epitope-based DNA vaccine strategy shows potential as a novel approach to preparing vaccines based on defined and conformation-dependent protective epitopes.

ACKNOWLEDGMENTS

The University of Southampton Strategic Development Fund and Cancer Research UK supported this work.

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Editor: F. C. Fang