The contribution of DNA immunization to the study of the immune response against Neisseria meningitidis

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REPORT

Introduction

Approximately 1.2 million cases of meningococcal disease are reported every year throughout the world, with an estimate of 170 000 deaths and a significant number of convalescents with permanent sequelae [1]. Although effective vaccines based on the individual polysaccharides from *Neisseria meningitidis* serogroups A, C, Y and W135 are currently available [1], similar efforts to obtain vaccines against serogroup B using its capsular polysaccharide have failed due to the low immunogenicity of this antigen in humans. Alternative approaches to the development of vaccines against this serogroup have focused on non-capsular surface antigens such as the outer membrane proteins (OMPs), which have been evaluated individually or as part of outer membrane vesicles (OMVs). The first study bringing information on the efficacy and safety of an OMV-based preparation took place in Cuba and evaluated the VA-MENGOC-BC® antimeningococcal vaccine [2, 3]. Nevertheless, even when OMVs induce protective immunity against different meningococcal strains, their immune responses can be improved by including new adjuvants, or by adding conserved antigens to vaccine preparations.

Genomic and proteomic techniques have recently been used for the identification of new low-abundance outer membrane proteins which are antigenically conserved among all isolates [4], although direct real-life applications of these results are not yet available. These antigens have been included in current efforts for the enhancement of available vaccine preparations, together with ongoing work on new immunization techniques, immunization routes, and an emphasis on the induction of cell-mediated responses against the meningococcus [5]. Within this context, the methodology of DNA immunization is uniquely suited for this purpose. DNA immunization is based on the direct inoculation of the gene coding for the vaccine antigen into the tissues of the host, in contrast with the conventional practice of inoculating the protein itself [6]. This technique potentially allows the evaluation of a large number of antigens in a given animal model, via methodologies such as Expression Library Immunization (ELI) [7]. In addition, DNA immunization stimulates all the effector branches of the immune system, yielding therefore a more integral picture of the host immune response. In this work, we show the results jointly obtained at the Department of Anti-Meningococcal Vaccines of the Center for Genetic Engineering and Biotechnology (CIGB) and the Laboratory of Molecular Biology at the Finlay Institute; demonstrating for the first time the use of ELI as a valid alternative for the study of the immune response against the meningococcus.

Construction of the pELI3.1 vector

In order to obtain an optimal (efficient and representative) arrayed genomic library of *Neisseria meningitidis*, we designed a new expression vector for DNA immunization, named pELI3.1 [8] (Figure 1). Heterologous expression from pELI3.1 in eukaryotic hosts is driven by the human Cytomegalovirus immediate/early promoter and transcriptional termination/polyadenylation sequences of the simian virus 40 t antigen. Among the new features of the pELI3.1 vector are: a) it incorporates the well known white/blue visual recombinant selection scheme based on beta galactosidase; b) includes a Kozak sequence before the first translational codon for optimized expression in eukaryotic cells; c) a multiple cloning site (MCS) expanded by adding *BamH* I, *Bcl* I and *Bgl* II restriction sites in the three open reading frames (ORFs), all of them compatible with the *Sau3*AI restriction enzyme; d) binding sites for the M13 universal primers flanking the MCS, designed for easy sequencing of DNA inserts; and e) the overall size of the plasmid was reduced to 3 892 bp to increase its capacity for the insertion of DNA fragments and for more efficient transfection.

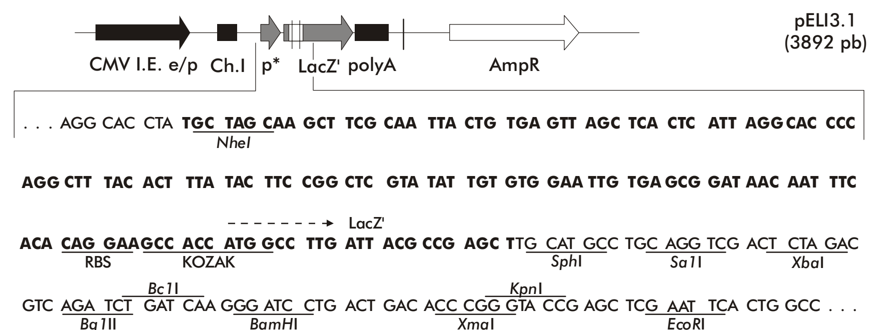

Figure 1. Diagram of the pELI3.1 vector. CMV I.E e/p- human cytomegalovirus immediate/early promoter; Ch.I- chimeric intron; p*- lactose operon promoter; LacZ'- LacZ alpha subunit; polyA- SV40t polyadenylation signals; AmpR- ampicillin resistance gene. The sequence of the multiple cloning site (MCS) is depicted, showing the restriction endonuclease recognition sequences and underlining the translational control signals for bacteria (RBS) and eukaryotic cells (Kozak).

1. Mitka M. New vaccine should ease meningitis fears. JAMA 2005;293(12): 1433-4.

2. Sotolongo F, Campa C, Casanueva GV, Fajardo EM, Cuevas I, González N. Cuban Meningococcal BC Vaccine: Experiences & Contributions from 20 Years of Appli-cation. MEDICC Review 2007;9(1): 16-22.

3. Sierra GV, Campa C, Varcacel NM, García 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(2):195-207.

4. Bernardini G, Braconi D, Martelli P, Santucci A. Postgenomics of *Neisseria meningitidis* for vaccines development. Expert Rev Proteomics 2007;4(5):667-77.

5. Pérez O, Lastre M, Lapinet J, Bracho G, Díaz M, Zayas C, *et al*. Immune response induction and new effector mechanisms possibly involved in protection conferred by the Cuban anti-meningococcal BC vaccine. Infect Immun 2001;69(7):4502-8.

6. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 2000; 18:927-74.

7. Barry MA, Howell DP, Andersson HA, Chen JL, Singh RA. Expression library immunization to discover and improve vaccine antigens. Immunol Rev 2004;199: 68-83.

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Construction of an arrayed genomic library of *N. meningitidis* and its evaluation in mice

After the obtention and evaluation of the new vector, an arrayed genomic library of *N. meningitidis* was constructed. Chromosomal DNA from strain CU385 was partially digested with *Sau3*AI (1U/mg DNA, 5 min at 37 ºC) and ligated in three separate reactions to pELI3.1 digested with either *Bgl*II, *BamH*I or *Bcl*I. TOP10 *Escherichia coli* cells were transformed with each ligation reaction, plated on selective media supplemented with X-Gal and IPTG, and incubated until the obtention of visible colonies. Subsequently, 6 000 white colonies were hand-picked and inoculated into 96-well plates containing 2xYT medium, followed by growth for 6 h, addition of glycerol to a final concentration of 15%, and storage at -70ºC. Replicate plates were prepared from each master plate, grown for 6 h, and used to inoculate 10 mixed liquid LB cultures with 600 clones each (the pre-growth period was introduced to avoid biasing the composition of the resulting population towards fast-growing individuals). Finally, total plasmid DNA was purified with pyrogen-free plasmid purification kits (Qiagen, Canada) from each culture, and a pool of the 10 resulting preparations was used to immunize Balb/c mice intramuscularly with four doses of 100 mg of DNA each. Specific IgG antibody responses were detected by Western blot and ELISA in the sera of mice immunized with the complete library, which additionally showed bactericidal activity titers of 1:16 [8] (the induction of bactericidal antibodies is the gold standard used by international regulatory agencies to evaluate the effectiveness of anti-meningococcal vaccines, with titers higher than 1:8 usually regarded as positive). The inguinal nodes were extracted from three animals from each group and used to obtain lymphocytes for lymphoproliferation studies against meningococcal whole-cell lysates (5 µg/well) or OMVs (5 µg/well). The groups immunized with the DNA library showed stimulation indexes higher than 3 (5.4 ± 0.9 against lysates and 5.5 ± 1.7 against OMVs), as did the groups immunized with the Cuban commercial vaccine (5.1 ± 1.0 against lysates and 3.5 ± 0.8 against OMVs). This result demonstrated the existence of meningococcal polypeptides with immunostimulatory effects within the library, and guided further fractionation efforts for the identification of functional clones.

Fractionation of the arrayed genomic library

Plasmid DNA from each of the 10 library fractions described above was used to immunize Balb/c mice, following a schedule similar to that employed for the complete library. Three of the six sub-libraries (L6, L8 and L9) induced antibody responses with bactericidal activity and one of them (L8) also conferred protection (*i.e.*, a significant reduction in the number of colonies) in the infant rat bacteremia model. Some of the sub-libraries induced IgG antimeningococcal antibodies in serum detectable by Western blot, and specially, sera from mice immunized with the L8 sub-library recognized a well-defined band in OMVs preparations. Therefore, sub-library L8 was further fractionated into 6 sub-libraries of 95 clones each as previously described, denominated L8A to F. Total plasmid DNA was purified from each fraction and used to immunize Balb/c mice, resulting in the induction of a bactericidal antibody response (1:8) for sub-library L8C. Additionally, when these six sub-libraries were combined in pairs and evaluated once again in an immunization schedule, the L8C-L8D combination induced the highest bactericidal titers and also conferred protection in the infant rat bacteremia model.

Identification of vaccine candidates against *N. meningitidis* by genomic immunization

In order to identify the antigens responsible for the bactericidal and protective antibodies described above, plasmid DNA from 190 individual clones of the L8C and L8D sub-libraries was sequenced and analyzed by bioinformatic procedures. Based on proper reading phase and insertion of the identified sequences in pELI3.1, 20 out of the 190 clones were presumed to be able to express meningococcal polypeptides (Table 1) and were chosen for further analysis [9]. The antigens predicted to be encoded by these clones corresponded to 8 OMPs, 1 periplasmic and 11 cytosolic proteins (Table 1). One of these proteins, named clone 3E9, is a typical conserved OMP, and therefore, an attractive vaccine candidate.

Evaluation of a group of plasmids as vaccine candidates against *N. meningitidis* in mice

The evaluation of the vaccine potential of the 20 putative antigens described above was carried out by inoculating mice with four doses of pooled plasmid DNA (100 µg), administered at intervals of 3 weeks, representing combinations of the clones previously identified by the ELI selection method. One group was immunized with the L8C-L8D combination (L8CD, all 20 plasmids), two with the positive clones from each plate (L8C and L8D), one with the 8 clones coding for OMPs (L8-OMP) and the remaining group was immunized only with clone 3E9. A negative control group immunized with the empty vector was also included (Table 1). Although bactericidal activity was detectable only in sera from animals immunized with the L8CD pool or with the clones from sub-library L8D, the elicited immune response recognized not only the homologous strain, but also a strain from a different serogroup. These results were verified by an *in vitro* flow cytometry assay for human complement deposition at the bacterial surface, and also in an infant rat model of passive immunization, where the sera from groups L8CD and L8D conferred protection to the animals. Finally, the immunized mice from the scheme above were challenged with approximately 2 x 10^7 colony-forming units (c.f.u.) of the homologous meningococcal strain (Figure 2A). The individuals from the L8CD combination and the group receiving clone 3E9 were partially protected (more than 50% survival), in contrast with the other groups (survival rates < 20%) [9].

Prime-boost strategy for studying the immune response against *N. meningitidis*

To evaluate this alternative, the gene *porA* from the meningococcal strain CU385 strain was tested in diffe-

8. Yero CD, Pajón FR, Caballero ME, Cobas AK, López HY, Farinas MM, *et al*. Immunization of mice with *Neisseria meningitidis* serogroup B genomic expression libraries elicits functional antibodies and reduces the level of bacteremia in an infant rat infection model. Vaccine 2005; 23(7):932-9.

9. Yero D, Pajón R, Pérez Y, Farinas M, Cobas K, Díaz D, *et al*. Identification by genomic immunization of a pool of DNA vaccine candidates that confer protective immunity in mice against *Neisseria meningitidis* serogroup B. Vaccine 2007; 25(28):5175-88.

Table 1. Antigens identified by immunization with DNA fragments from a genomic library of *Neisseria meningitidis* strain CU385

Clone	Source plate	Name of the encoded protein and putative function as determined by sequence homology	Cellular location	Immunization groups*
3B1	L8C	Transposase IS5	Cytoplasm	A B
3E9	L8C	Membrane lipoprotein	Outer membrane	A B C D E
3F1	L8C	ClpA, ATPase with chaperone activity	Cytoplasm	A B
3F4	L8C	YCII domain with enzyme activity	Cytoplasm	A B
3F10	L8C	Putative protein	Unknown	A B
3G3	L8C	Putative protein	Outer membrane	A B D
3G7	L8C	HesB domain involved in nitrogen fixation	Cytoplasm	A B
3G9	L8C	Fosfopantetein adenyltransferase	Cytoplasm	A B
4A1	L8D	Outer membrane ferric coprogen receptor	Outer membrane	A C D
4A6	L8D	LuxS, population level self-inducible sensor	Cytoplasm	A C
4A8	L8D	MurC, UDP-N-acetylmuramate-alanine ligase	Cytoplasm	A C
4A10	L8D	ResB protein, cytochrome C synthesis	Innermembrane	A C
4A11	L8D	AdhC, Zn++-dependent alcohol dehydrogenase	Cytoplasm	A C
4D4	L8D	Adhesin AidA, Type V secretion pathway	Outer membrane	A C D
4E5	L8D	Porin PorA	Outer membrane	A C D
4F2	L8D	Hemagglutinin	Outer membrane	A C D
4F11	L8D	Guanosine polyphosphate pyrophosphohydrolases /synthetases	Cytoplasm	A C
4G2	L8D	Toxin RTX, Ca++-related binding protein	Outer membrane	A C D
4H1	L8D	DapB, dihydrodipicolinate reductase	Cytoplasm	A C
4H10	L8D	Co++ transport system type ABC	Periplasm	A C D

*The letters stand for the clones included on each immunogen. A: mixed group of 20 clones; B, C: groups of clones from plates L8C and L8D, respectively; D: group of 8 membrane proteins; E: group treated with the plasmid derived from clone 3E9.

rent administration strategies. The priming capacity of DNA immunization, as the primary stimulus for cells of the immune system, was demonstrated by a strategy that combines a priming DNA followed by a boost with the protein antigen. After three inoculations with a plasmid coding for PorA (pELI-PorA), two groups of mice were boosted either with OMVs (containing the PorA on its native conformation) or recombinant PorA protein, respectively. Animals receiving the OMVs showed higher antibody titers against the PorA protein ($p < 0.05$) and the OMVs ($p < 0.05$), compared to the groups primed with the empty vector or phosphate-buffered saline. This result suggests that it is possible to increase the immune response against a meningococcal protein through an immunization schedule combining DNA and protein immunization against the same antigen. There were no differences in antibody titers for the group boosted with PorA in comparison with the control groups, although there were differences in their IgG antibody subclass profiles (the IgG2a/IgG1 ratio for anti-PorA antibodies was 4.5 for the animals primed with pELI-PorA, but approached unity for the control groups). Our results agree with previous findings from other groups on the use of DNA immunization to favor the induction of effector, rather than memory B cells; and constitute the first study combining DNA and protein immunogens in the same immunization schedule against *N. meningitidis*. The higher bactericidal titers observed in animals previously immunized with pELI-PorA are probably due to the preponderance of PorA-specific class IgG2a antibodies in these groups. The bactericidal titers increased significantly (two logs higher) two weeks after administering the PorA boosting dose. The results, as a whole, demonstrate that it is possible to modify the immune response against a meningococcal antigen by combining sequential plasmid DNA and protein immunizations.

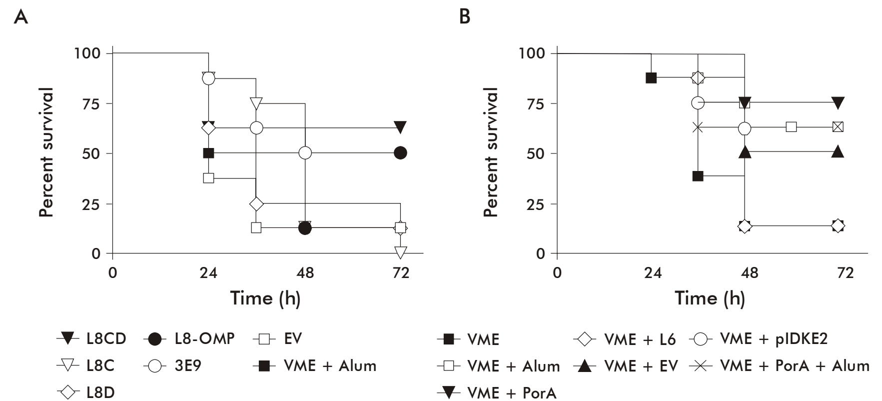

Figure 2. Results of challenge experiments on mice immunized with the group of clones identified by ELI (A) or with combinations of meningococcal outer membrane vesicles (OMVs) and DNA fragments (B). Mice were challenged after immunization with ~2 x 10^7c.f.u. of strain CU385, and survival was monitored for three days. EV: empty pELI3.1 vector used as negative control; L8-OMP: combination of all the clones coding for putative outer membrane proteins; L8C and L8D: sub-libraries C and D; L8CD: combination of the L8C and L8D sub-libraries;3E9: clone coding for a membrane protein isolated from the L8C sub-library; pIDKE2: plasmid coding for antigens of the hepatitis C virus, unrelated to the assay system; OMP: outer membrane protein; PorA: plasmid coding for porin; L6: sub-library inducing antibodies with bactericidal activity.

Prime-boosting as a screening strategy for vaccine candidates

The prime-boost strategy was explored once again by immunizing mice with random combinations of clones from a genomic library of *N. meningitidis* strain CU385, followed by a boost with OMVs. Sera were collected from the animals and their antibody titers were evaluated against OMVs. Although no differences in antibody levels were detected, there was significant variation in bactericidal activity. Two combinations induced bactericidal and protective immune responses against *N. meningitidis* in animal models, and their Western blot profiles (against meningococcal proteins) were different from those of animals primed with the empty

vector. Based on these results, a new strategy for screening vaccine candidates was proposed, based on the combination of priming with genomic libraries and boosting with proteins from the target pathogen [10].

Adjuvant effect of different DNA molecules for the OMVs of *N. meningitidis*

These experiments examined the potential of the vectors obtained in this work for their use in formulations where plasmid DNA is co-administered with OMVs. Balb/c mice were immunized with three doses containing equal amounts (5 mg each) of non-adjuvanted OMVs alone or in combination with either the pELI-PorA vector, a plasmid coding for a non-related antigen (pIDKE2), a subgroup of clones from the genomic L6 sub-library, or the empty vector (pELI3.1). Mice immunized with OMVs adjuvanted in alum gel were used as positive control. Significant differences were detected in antibody titers as measured by indirect ELISA against OMVs or PorA among the groups immunized with OMV-plasmid DNA combinations in comparison to the control group receiving non-adjuvanted OMVs. The most straightforward interpretation of this result is that plasmid DNA has an adjuvant effect on OMVs, in agreement with the fact that while the group receiving non-adjuvanted OMVs had a preponderance of IgG1 meningococcal-specific antibodies, the groups receiving OMV-plasmid DNA combinations showed a balanced IgG2a/IgG subclass ratio (as also seen in the mice immunized with OMVs adjuvanted in alum). The bactericidal assay results largely agreed with this interpretation. No differences in serum bactericidal activity were detected between the positive control (OMVs adjuvanted in alum) and the animals immunized with the plasmid DNA-OMV formulations after the third dose, contrasting with the absence of bactericidal antibodies in the group immunized with non-adjuvanted OMVs. These levels of bactericidal activity, in the groups being co-administered plasmid DNA with OMVs, did not depend on the identity of the plasmid after the second dose. Further data on the functional activities of the responses elicited by plasmid DNA-OMVs combinations was gathered by challenging the animals one month after the last dose with 2×10^7 c.f.u. of the homologous strain. Seventy five percent of the mice immunized with OMVs + pELI-PorA survived, while 90% of the animals receiving non-adjuvanted OMVs died; additionally, there was a survival rate of 50% for the animals immunized with OMVs+pELI3.1 or alum-adjuvanted OMVs (Figure 2B). These results represent the first demonstration, to our knowledge, of the adjuvant properties of plasmid DNA for meningococcal OMVs.

Conclusions

The results shown in this work demonstrate that it is possible to modulate the immune response against protein antigens and OMVs of *N. meningitidis* by combining plasmid DNA immunization strategies. Our data highlights the potential of DNA immunization as a tool for the screening of novel meningococcal vaccine candidates, especially when used within prime-boost immunization regimes.

10. Yero D, Pajón R, Caballero E, González S, Cobas K, Farinas M, *et al.* A novel method to screen genomic libraries that combines genomic immunization with the prime-boost strategy. FEMS Immunol Med Microbiol 2007;50(3):430-3.