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Characterization of the key antigenic components of pertussis vaccine based on outer membrane vesicles

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ABSTRACT

Pertussis has resurged during the last two decades in different countries. In particular in the 2010–2013 period large outbreaks were detected in US, Australia, UK and The Netherlands with significant mortality in infants. The epidemiological situation of pertussis points out the need to develop new vaccines and in this regard we previously developed a new vaccine based on outer membrane vesicles (OMVs) which have been shown to be safe and to induce protection in mice. Here we have further investigated the properties of OMVs vaccines; in particular we studied the contribution of pertussis toxin (PTx) and pertactin (Prn) in OMVs-mediated protection against pertussis. PTx-deficient OMVs and Prn-deficient OMVs were obtained from defective *Bordetella pertussis* mutants. The absence of PTx or Prn did compromise the protective capacity of the OMVs formulated as Tdap vaccine. Whereas the protective efficacy of the PTx-deficient OMVs in mice was comparable to Prn-deficient OMVs, the protective capacity of both of them was significantly impaired when it was compared with the wild type OMVs. Interestingly, using OMVs obtained from a *B. pertussis* strain which does not express any of the virulence factors but expresses the avirulent phenotype; we observed that the protective ability of such OMVs was lower than that of OMVs obtained from virulent *B. pertussis* phase. However, it was surprising that although the protective capacity of avirulent OMVs was lower, they were still protective in the used mice model. These results allow us to hypothesize that OMVs from avirulent phase shares protective components with all OMVs assayed. Using an immune proteomic strategy we identified some common components that could play an important role in protection against pertussis.

1. Introduction

Despite relatively high vaccination rates, in recent years the respiratory disease named pertussis or whooping cough has came roaring back to infect people in high numbers. Indeed, pertussis is now recognized as a frequent infection not only in newborn and infants, but also in adults [1–3]. The increase in pertussis cases has been mostly attributed to waning vaccine-induced immunity, the switch from whole cell vaccines to acellular vaccines (aP) and pathogen adaptation [4]. Despite the causes, the epidemiological situation has led to intensified research on the design of new vaccines capable of conferring both long-lasting immunity and protection against different strain genotypes. Recently, we have demonstrated that outer membrane vesicles (OMVs) derived from *Bordetella pertussis* can protect against intranasal pertussis challenge when administered in a mouse infection model [5]. OMVs seem to have important advantages in vaccine development over the currently used aP since they are capable of conferring both long-lasting immunity and protection against different strain genotypes, eliciting IFNγ response, that have been associated with a better protective profile [6,7]. To further study the mechanisms involved in OMVs-mediated protection and in particular the contribution of some virulence factors in the protective efficacy, we investigated the role of pertussis toxin (PTx) and pertactin (Prn), which are present in the OMVs, derived from wild type *B. pertussis* [22]. PTx and Prn have shown to be protective antigens

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of *B. pertussis* and are used as essential components of acellular pertussis vaccines [8–10]. The PTx is a multimeric AB subunit protein in which A monomeric subunit expresses toxic activity through ADP ribosylation of the membrane-bound G_i-likeproteins, leading to the blockage of certain transmembrane signaling processes and eventually to cell intoxication. The B pentameric subunit binds receptors on the surface of eukaryotic cells and allows the toxic A subunit to reach its intracellular targets [11]. Several studies indicate that pertussis toxin possesses adjuvant properties able to potentiate local and systemic Ab responses, enhancing IgE, IgA, and IgG production [12,13] and promoting Th1- and Th2-type responses to co-administered Ags [14,15]. More recently, Nasso et al. using a genetically detoxified variant of pertussis toxin have shown that it promotes a Th1/Th17 response by an IL10 dependent mechanism [16].

PRN is a surface protein, which contains a RGD motif (Arg–Gly–Asp) involved in the attachment of *B. pertussis* to mammalian cells [11]. Anti-pertactin antibodies were found to be crucial for *B. pertussis* phagocytosis [17]. These data are consistent with field studies showing that the levels of pertactin antibodies correlate with protection [18].

In this study, we used PTx or Prn-deficient OMVs to analyze their immunogenicity, and protective efficacy in the well-accepted murine model of *B. pertussis* infection. Moreover, we analyzed whether the expression of all virulence factors is necessary to induce protection against pertussis. To this aim, we obtained OMVs from a *B. pertussis* strain blocked in the avirulent phase, a phase well characterized by the absence of all the virulence factors dependent on the BvgAS two component system but still able to express a whole set of new proteins known as avirulence factors.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. pertussis Tohama phase I strain, the Argentinean clinical isolate *Bp*106 [7,19] and the mutant strains defective in PTx [20], Prn [21] or BvgS protein [22] expression were used throughout this study. The *B. pertussis* strains were grown on Bordet Gengou (BG) agar medium (Difco, Houston, US) supplemented with 10% (v/v) defibrinated sheep's blood (BGA plates) [19].

2.2. Isolation of outer membrane vesicles (OMVs)

OMVs from bacterial cells we obtained according to the previously described method [23,24]. OMVs were negatively stained and examined by transmission electron microscopy as explained elsewhere [23,24]. Protein content was estimated by the Lowry method [25] using bovine serum albumin as standard.

2.3. Cloning and expression of the recombinant subunit A of pertussis toxin (PTxA), Fimbriae 2 (Fim2) and GroEL like protein

As previously stated for the PTxA subunit [7], the PCR product of each coding sequence obtained using Pfx Platinum polymerase (0.5 units, Invitrogen) was cloned into pET-TOPO 200 vector according to the manufacturer's instructions (Invitrogen). The recombinant clones were confirmed by PCR and sequenced. The recombinant pET-TOPO 200 vectors containing each coding sequence were first transformed into TOP10 *Escherichia coli* competent cells and then into expression host cells, BL21Star. For protein expression, selected clones were grown in LB medium containing km (50 μg/ml) and IPTG 1 mM.

Each recombinant protein was purified using Ni-NTA columns (Invitrogen) equilibrated in denaturing lysis buffer (Invitrogen) according with the manufacturer's protocols. Recombinant protein purity was judged by SDS-PAGE. The identity of the expressed protein was confirmed by MALDI-TOF mass spectrometry [7]. Search and identification of peptides were performed with a licensed Version of MASCOT software (Matrix Science at http://matrixscience.com), in a database containing the 3436 accession number entries derived from the complete *B. pertussis* genome sequence (downloaded from http://www.ncbi.nlm.nih.gov/). The MASCOT search parameters: (i) species, Bacteria (Eubacteria); (ii) allowed number of missed cleavages (only for trypsin digestion) 1; (iii) variable post-translational modifications, methionine oxidation; (iv) fixed modifications, carbamidomethylation; (v) peptide tolerance, ±50 ppm; (vi) peptide charge, +; and (vii) monoisotopic peptide masses, were used to search the database, allowing a molecular mass range for 1-DE analyses of ±15%. Only significant hits as defined by MASCOT probability analysis were considered. The sequence coverage % for the identified proteins was higher than 37%.

2.4. Production of a polyclonal serum against His6-PTx S1, His6-Fim2, His6- GroEl and Prn

To obtain specific sera against each recombinant protein and Prn (NIBSC), groups of 3 mice obtained from the Instituto Biológico Argentino (Biol. SAIC, Argentina) were immunized intraperitoneally twice, at intervals of 14 days, with 5 μg of each immunogen each time. $Al(OH)_3$ was used as an adjuvant. Fourteen days after the last immunization, mice were bled, and the serum was separated in order to assess by immunoblotting the presence of specific antibodies against the recombinant proteins.

2.5. Gel electrophoresis and immunoblots

OMV proteins were separated by SDS-PAGE and then transferred onto PVDF (Immobilon P, Millipore) at constant voltage (100 V). After the transfer, PVDF membranes were probed with either a polyclonal anti-PTx S1 antibody (diluted 1:300), an anti-Prn antibody (diluted 1:500), an anti-Fim antibody (diluted 1:500) or an anti GroEl (diluted 1:500), followed by incubation with anti-mouse IgG conjugated with alkaline phosphatase (AP) at a 1:1000 dilution. Nitroblue tetrazolium (NBT)—5-bromo-4-chloro-3-indolylphosphate (BCIP) was used as the AP substrate according to the manufacturers protocol (Promega).

2.6. Formulation of acellular vaccines containing OMVs, $Tdap_{OMVs}$

The vesicle preparations were detoxified with formalin (0.37% at 37 °C overnight). To use the OMVs from wild type bacteria or their deficient mutants as a combined acellular vaccine, vesicles (1.75 μg of total OMVs protein), were formulated with diphtheria (1 to 3 Lf/dose with an output of 0.1 UIA/ml serum) and tetanus toxoids (5 to 7 Lf/dose with a power greater than or equal to 2 UIA/ml serum) in the amounts described below, besides aluminum hydroxide did not exceed 1.25 mg/dose.

2.7. Active immunization and intranasal challenge

Animal protocol using female BALB/c mice (4 weeks of age) was performed as previously described [19]. Groups of 5 mice were immunized systemically (i.p) with Tdap vaccines containing specific OMVs (1.75 μg of total OMV protein that contains approximately 47.1 ng ± 11.2 ng of the complete PTx protein) or 1/10 human dose (HD) of commercial TdaP (Boostrix™ that contains as pertussis components PTx 8 μg, FHA 8 μg and Prn 2.5 μg per human dose) using a two-dose schedule over a period of 2 weeks. Mice were challenged 2 weeks after the second immunization by nasal inoculation with a sublethal dose (10^6–10^8 CFU 40 μl^{-1}) of Tohama phase I.