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Adjuvant and immunogenic activities of the 73 kDa N-terminal α-domain of BrkA autotransporter and Cpn60/60 kDa chaperonin of *Bordetella pertussis*

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Abstract

A soluble fraction obtained from *Bordetella pertussis* was evaluated as adjuvant for the pertussis component of the Diphtheria-Pertussis-Tetanus (DPT) vaccine. High levels of antibodies were induced, and a 78% protection rate of mice challenged with live *B. pertussis* was observed. Two proteins were identified as the 73 kDa N-terminal α-domain of BrkA autotransporter protein and the Cpn60/60 kDa chaperonin. Both stimulated antibodies against pertussis and induced a 42% protection rate against the challenge. IgG1 and IgG2a were stimulated suggesting that the immune response could be modulated to produce Th1 or Th2.

Keywords: *Bordetella pertussis*; Adjuvant; Vaccine

1. Introduction

The development of new vaccines with purified proteins or peptides requires the use of adjuvants since these purified molecules are frequently poor immunogens. Adjuvants are essential components to enhance the immunogenicity of vaccine antigens. Despite two centuries of vaccine use, only a few adjuvants are licensed for human use [31]. Aluminum compounds are almost the only adjuvants approved for use in humans, in spite of their limitations and the possibility to cause toxic and undesirable effects [17]. In addition, these compounds direct the immune response almost exclusively towards the Th2 arm [16,23]. The search for alternative adjuvants is increasing rapidly and these products must ensure appropriate antigen uptake, transport and presentation to stimulate an adequate immune response [24,27,31,32]. Moreover, for vaccine strategies optimisation, it is important to understand the mechanisms by which detection and processing of particles by the immune system can be improved [3,21,41].

Extracts of bacterial origin are well known immunomodulatory substances that can stimulate the immune responses increasing the antibody production and stimulating T-cells and macrophages [8]. *Bordetella pertussis*, the gram-negative pathogen that causes whooping cough, is a promising candidate since it produces several components acting on the immune system of the host [25,42].

BrkA is a virulence factor of *B. pertussis* that confers serum resistance to killing by the classical pathway of complement and is involved in adherence and invasion [13]. BrkA is an autotransporter protein, member of a functionally diverse family of outer membrane proteins in gram-negative

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bacteria that mediate their own export across the bacterial outer membrane [10,20]. It is expressed as a 103 kDa precursor that is processed during secretion to yield a 73 kDa N-terminal passenger α-domain and a 30 kDa C-terminal transporter β-domain [38]. Although cleaved, the 73 kDa BrkA passenger domain remains tightly associated with the bacterial surface and is not detected in *B. pertussis* culture supernatants [28,29]. The C-terminal domain has the ability to form a pore in lipid bilayer membranes [38]. A conserved region in this domain was identified and seems to be required for folding of the BrkA passenger [30].

Adjacent to BrkA, and transcribed in the opposite direction of the complementary strand, is the gene for Cpn60, a 60 kDa heat-shock protein (HSP), member of the chaperonin 60 family of highly conserved proteins which are involved as molecular chaperones in many essential cellular functions and additionally, in cell–cell signaling [35]. These proteins are known to be one of the most immunogenic bacterial antigens and have been implicated in immune regulation, both at the level of innate and adaptive immunity [11,40], and also in the pathology of infectious and immune diseases [34]. Evidence now suggests that self-reactivity is anti-inflammatory and can attenuate autoimmunity and organ transplant rejection [33]. HSPs can induce T-cell regulation and the secretion of regulatory cytokines such as interleukin-10 and promote a switch from a proinflammatory cytokine-secretion profile to a regulatory cytokine-secretion profile [12]. *B. pertussis* Cpn 60 was cloned and purified and found to be structurally related to the Cpn 60 *Escherichia coli* GroEL [4,14]. Notwithstanding, in spite of significant sequence conservation among these proteins, diverse Cpn60 can bind to several cell-surface receptors and express very different biological activities [22].

We have been studying the immunogenic and adjuvant properties of a *B. pertussis* soluble fraction (BS) obtained from bacterial whole cells [5,6]. In the present study, we characterized BS components involved in the modulation of the immune response. We purified two *B. pertussis* proteins, the 73 kDa N-terminal α-domain of BrkA autotransporter and the Cpn60/60 kDa chaperonin, and evaluated their immunogenic and adjuvant activities. A simple method is proposed to obtain the soluble fraction and purified proteins. Therefore, these proteins are good candidates to be explored as adjuvants for inclusion in vaccination protocols.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and BS fraction preparation

B. pertussis strains 137 and 143, serotypes 1,2,3,5,6 (NIH, Bethesda, USA), were obtained as bacterial suspensions (Setor de Vacinas Bacterianas, Instituto Butantan, São Paulo, SP, Brazil) by discontinuous growth in Cohen & Wheeler (CW) medium [7], and were concentrated by precipitation with 5 N citric acid and inactivated in a water-bath at 56 °C for 60 min. Thimerosal was added to a final concentration of 0.015%. The bacterial cell mass was obtained by centrifugation at 5000 × *g* for 30 min, and the pellet was lyophilized. For the preparation of BS fraction, the pellet was further reconstituted with ultrapure water (Milli Q, Millipore) (1:50), shaken for 6 h at room temperature (RT) and remained overnight at 4 °C. The suspension was then centrifuged at 7500 × *g* for 30 min. The procedure was repeated three times and the supernatants collected, pooled and filtered through a 0.45 μm membrane.

2.2. Protein quantification

All protein determinations were performed with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using a standard curve prepared with bovine serum albumin.

2.3. Separation of BS components

Fraction BS was applied to a Mono Q Sepharose (Amersham, Biosciences) (0.5 mg protein per ml resin) previously equilibrated with 25 mM Tris–HCl, pH 8.0. Proteins were eluted with a NaCl gradient (0–1 M) in 25 mM Tris–HCl, pH 8.0, in 0.5 ml fractions at a flow rate of 1 ml/min. Elution was monitored at $A_{280\,nm}$. The fractions showing the same peak profile were pooled and eluted again under the same chromatography conditions. Chromatography was performed in a FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden). The eluted fractions (5 μg protein) were concentrated (Centrivap Concentrator, Labconco) and reconstituted in sample buffer (62.5 mM Tris, 2% SDS, 20% glycerol, 0.001% bromophenol blue, pH 6.8), heated 5 min at 100 °C and centrifuged. Samples were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (T = 12.5% in 0.75 M Tris, 0.2% SDS, pH 8.8) and electrophoresis was performed at 60 mA in 25 mM Tris, 0.192 M glycine, 0.1% SDS, pH 8.3. The gel was stained with silver nitrate and fractions showing a similar molecular mass profile were pooled, concentrated by centrifugation at 440 × *g* in Centrifugal Ultrafree-20 tubes, 10 kDa (Millipore), and their protein concentration was evaluated. Four pools were formed (P1, P2, P3, P4), and used for immunization of mice.

2.4. Purification of B. pertussis proteins

Fraction BS was applied to a Q Sepharose (Hitrap Q Sepharose HP, Amersham Biosciences) column (3.5 mg/ml) previously equilibrated with 25 mM Tris/HCl, pH 8.0. Proteins were eluted using the same conditions as described above, in fractions of 1.5 ml at a flow rate of 5 ml/min. Elution was monitored at $A_{280\,nm}$. Fractions of the peak corresponding to pool 4 (P4) were pooled, concentrated by centrifugation in Ultrafree-20 tubes as described above, and their protein concentration was evaluated. Chromatography was performed in an Äkta system (Amersham Pharmacia Biotech,