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PURIFICATION AND PROTECTIVE EFFICACY OF RE-NATURED C-TERMINAL DOMAIN (P.30) OF PERTACTIN AUTOTRANSPORTER PROTEIN OF *BORDETELLA PERTUSSIS*

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Abstract

Members of the *Bordetella pertussis* autotransporter family are grouped together on the basis of homology at their C-termini. The full pathogenic potential of the *B. pertussis* is partly contributed by the members of its autotransporter family, which are usually either exported to the bacterial cell surface or secreted into the external environment. The β-barrel (C-terminus) forming domain plays a key role in surface localization of the virulence associated passenger domains of autotransporter proteins. The protective capacity of the C-terminus (P.30) of *B. pertussis* autotransporter protein pertactin was studied in mouse protection studies.

Introduction

B. pertussis is the causative agent of highly infectious and acute childhood disease known as whooping cough with severe clinical manifestations (Hewlett. 1995). Effective killed whole-cell vaccines against *B. pertussis*-mediated disease have been used in developed countries for more than 50 years and have led to dramatic reductions in morbidity and mortality. However, the protection afforded by the whole-cell vaccine comes at the cost of its associated reactogenicity ranging from local and systemic reactions to permanent brain damage and death as suggested by previous studies by Cherry *et al*., 1988; Griffith, 1989; Hodder and Mortimer, 1992. These problems of reactogenicity have acted as a stimulus for the development of non-toxic efficacious vaccines in the form of acellular vaccines.

The contemporary research is focused on the identification of new protective antigens that would be efficacious and non-toxic (Preston & Maskell, 2002). Various pertussis virulence factors have been shown to be more or less protective when used in an acellular vaccine e.g. pertussis toxoid, filamentous haemagglutinin, pertactin, and fimbriae (Trollfors *et al.*, 1995). The safety and the extent of immunogenicity afforded by these vaccines paved the way for their consideration as candidates for booster immunization of older children and adults to control pertussis more effectively in populations (Keital and Edwards, 1995; Cherry *et al*., 1998).

The autotransporters are a growing family of extracellular proteins, found in many Gram-negative bacteria that have many different functions but appear to have the same mechanism of export (Henderson *et al.*, 2004). The ability of the *B. pertussis* purified tracheal colonization factor (TCF), bordetella resistance to killing (BrkA) and virulence associated gene 8 (Vag-8) autotransporter proteins in providing protection against the colonization of *B. pertussis* in the respiratory tract of mice is less clear (Fernandez and Weiss, 1994; Finn and Stevens, 1995; Finn and Amsbaugh, 1998).

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However, the protective properties of mature PRN (P.69) have been well documented across the *Bordetella* spp., as the immunisation with vaccines containing PRN, including recombinant PRN, and attenuated *S. typhimurium* expressing PRN, provides protection against *B. pertussis* infection in mice and *B. bronchiseptica* infection in mice and pigs (Charles *et al*., 1991; Roberts *et al.*, 1992; Mills *et al*., 1998). Therefore, autotransporter PRN is known to be an important protective component of many current acellular vaccines. However, there is increasing evidence of sequence variations of at least two important constituents of current acellular vaccines e.g. PRN and PTX in *B. pertussis* strains circulating in the population (Mooi *et al*., (1998). This has been suggested to be due to the selective pressure imposed by current vaccines (Gerlach *et al.,* 2001) and this situation has prompted the search for additional protective antigens that will be useful in enhancing the protective efficacy of the new generation of acellular pertussis vaccines.

Pertactin (P.93) is known to produce two fragments, an N-terminal passenger domain P.69 and a C-terminal (β-domain) domain P.30, after processing in *B.* pertussis (Junker *et al*., 2006). The β-domain of autotransporter generally consists of 250-300 amino-acid residues. From Bioinformatics analysis it has been predicted that the β-barrel of most autotransporter exhibit 14 antiparallel amphipathic strands consisting of 9 to 12 residues (Roberts *et al.*, 1992**;** Boursaux-Eude *et al*., 1999)**.** Moreover, there is a lack of knowledge regarding our understanding due to their membrane embedded nature. In addition, there is no experimental evidence available regarding the potential of the C-terminus of PRN (P.69) in providing any protection in animal models. Still exact shape of and conformation of the autotransporter β-barrel is subject to speculation hopefully this should be resolved in the near future through current efforts which are being made by several research teams to crystallize this domain.

The purified de-natured C-terminal domains of PRN, TCF and BrkA from *B. pertussis* outer membranes were not found to be protective in the intracerebral mouse protection test in previous mouse protection study by our group. This study was carried out to advance our understanding one step further regarding the protective ability of re-natured beta-barrel forming domain of autotransporter pertactin of *B. pertussis.*

Materials and Methods

Bacterial strains & plasmids: The details of bacterial strains and plasmids used in this study are given in table 1. The media used in the study for *E. coli*, which was routinely grown at 37°C, were Luria Bertani (LB) broth (Sigma) Luria Bertani agar (Sigma).

Urea extraction of inclusion bodies: The bacterial culture expressing recombinant proteins centrifuged at 5,000-x *g* for 15 min (Sorvall Superspeed centrifuge) and pellet resuspended in 20 ml of Buffer A (Sodium phosphate; 50mM, pH 7.4: Sucrose 5%). The suspended pellet disrupted using a One-Shot cell disrupter and the resultant lysate was centrifuged at 17,000-x *g* for 30 min to allow inclusion bodies to be pelleted. Such pellets were resuspended in 10 ml of Buffer B (8M urea, Sodium phosphate; 50mM, pH 7.4: Sucrose 5%) and inclusion bodies solubilised, with tube rotation, for 24 h or until the suspension cleared. A final ultracentrifugation step of 100,000-x *g* for 3 h was performed and the supernate containing urea-soluble protein was retained and stored at -20°C.

Table 1. The *E. coli* strains used in this study.

Strain	Genotype/Phenotypes	Source/Remarks
E. coli M15p (REP4)	Nal^s, Str^s, Rif^s, Thi^-, Ara^+, Gal^+, Mtl^-, $RecA^+$, Uvr^+, Lon^+	Qiagen; Recombinant protein expression strain
E. coli TOP10F'	F'{*lacI*^{q}, *Tn10* (Tet^R)} *mcrA* Δ (*mrr-hsd*RMS-*mcr*BC), ϕ80*lacZ*ΔM15, Δ*lacX*74, *deoR*, *recA*1, *araD*139 Δ(*ara-leu*)7639, *galU*, *galK*, *rspL*(Str^R), *endA*1 *nupG*	Invitrogen; General transformation strain
E. coli DH5α	*supE*44, Δ*lacU*169 (ϕ80*lacZ*ΔM15) *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	Infection and Immunity, University of Glasgow; General transformation strain

Table 2. Plasmids used or created during this study.

Plasmid Name	Comments	Source/Remarks
pET33b(Bp5CT1)	*bap-C* C-terminal region cloned into pET33b: expression construct for Bap-C, C-terminus	This study
pET33b(PCT1)	*prn* C-terminal region cloned into pET33b: expression construct for Prn C-terminus	This study
pET33b(TCT1)	*tcf* C-terminal region cloned into pET33b: expression construct for Tcf C-terminus	This study
pET11a(BCT1)	*brkA* C-terminal region cloned into pET11a: expression construct for BrkA C-terminus	This study

Protein quantification: The Bradford method was used to determine the protein content with bovine serum albumin as the standard. The absorbance at 562 nm was determined with a microtitre plate reader and protein concentrations estimated from a standard curve.

Dialysis: Dialysis tubing with a molecular weight cut-off of 12-14 kDa, stored in absolute ethanol, was boiled in distilled water immediately before use. The sample was loaded into the tubing, which was sealed with mediclips and placed into 1000 volumes of dialysis buffer (PBS or 1M urea in PBS). The dialysis was performed overnight with stirring at 4°C.

Preparation of antigens: Crude urea extracts or His-purified preparations were dialysed into 1 M urea (carboxyl terminal portion) or PBS (recombinant protein: P.69). These test antigens were diluted to 75 μg/ml in PBS containing 1.5 mg/ml alhydrogel. Purified P.69 antigen, extracted from *Pichia pastoris* (a gift from Prof. M. Roberts), was diluted to 75 μg/ml in PBS/alhydrogel. Further purification was performed according to the Ni-NTA spin kit (Qiagen), and the method was adapted from that described in the manufacturer's handbook.

Mouse protection test: The construct expressing pertactin C-terminal domain (pET 33b PCT1) was expressed in *E. coli* M.15 and the protein extracted from inclusion bodies by solubilisation in 8M urea. The crude urea extracts of P.30 were dialysed against 1 M urea, purified. The P-30 solution (500 μl) diluted 100 fold with 20mM sodium acetate (pH 5.8). After 12 h of incubation at 4°C, the aggregates were removed by centrifugation. The supernatant fraction was dialyzed against 20mM phosphate buffer, pH 6.0. The refolded protein was centrifuged at 12,000*g* for 30min to remove any insoluble material.

To prepare the vaccine doses re-natured P.30 or purified P.69 (recombinant P.69 purified from *Pichia pastoris*), 100 μg/ml of protein was mixed with an equal volume of alhydrogel Al $(OH)_3$. Thus each 0.2-ml volume contained 10 μg of protein and 10 μg of alhydrogel. A control for the experiment was to replace the antigen solution with an equivalent volume of PBS added to an equal volume of alhydrogel.

Immunisation: Randomised groups of female CD1 mice (Harlan Olac, Bicester, Oxfordshire, UK) aged 3-4 weeks (4 mice per group), were injected subcutaneously, under light halothane anaesthesia, with 10 μg of test antigen per mouse. One group of mice was vaccinated with renatured P.30 antigen whereas another group received the same amount of purified pertactin (P.69), a well-known component of acellular vaccines. A third group of mice received only alhydrogel in PBS. For each test group, a second dose, as above, was administered three weeks later. The weight of each group of mice was recorded prior to the vaccination and thereafter at regular intervals to check for any toxicity because of the antigen preparation.

Intranasal challenge: *B. pertussis* 18323 was grown as a lawn on BG plates in a humidified box at 37°C for 24h. The resultant growth was suspended in 1% casamino acids solution (Casein hydrolysate, Magnesium chloride $6H_2O$, Calcium chloride, Sodium chloride) and adjusted to 10 opacity units using the 5^{th} International Reference of Opacity i.e. approx. $2x10^9$ CFU/ml which was confirmed with plate counting method. Two weeks after the second vaccination the challenge suspension of *B. pertussis* 18323 containing ~$2x10^6$ CFU/ml was prepared and a sublethal dose of $1x10^5$ CFU in 50 μl volume was instilled intranasally to each mouse under light halothane anaesthesia. The weight of each group of mice was recorded prior to challenge as well as at regular intervals afterwards. Four mice/group were sacrificed at each sampling time on days 0, 3, 9 and 14 and the lungs and trachea were separately removed aseptically into sterile universal bottles. The nasal cavities of each mouse were located carefully by detaching the head of the animal first and then flushing sterile casamino acid through the nasal cavity (1ml each); the nasal washes were collected in sterile tubes.

Results

Mouse protection test using re-natured C-terminal (P-30) domain of pertactin: Since denatured P.30 fragment lacked the protective ability (Previous study: not shown), therefore re-natured preparation of the C-terminal 30 kDa moiety of PRN (P.30) was used to assess its protective capability if any.

A preparation of native purified PRN (P.69), being a known protective antigen in mice against respiratory tract colonisation, acted as a positive control. Alhydrogel alone (diluent) was included as a negative control. After being vaccinated twice subcutaneously with P.69, re-natured P.30 or with the diluent (alhydrogel) mice were challenged intranasally with a sub-lethal dose of the standard mouse-virulent *B. pertussis* strain 18323 (~$1x10^5$ CFU/mouse) and then sacrificed at intervals. Mouse lungs and tracheas were removed and nasal washes collected on post challenge days 0, 3, 9 and 14. Viable counts from individual mice were obtained and the raw data was extrapolated.

The results indicated (Not shown) that the mice treated with P.30 appeared to show no significant protection against nasal colonisation compared to the control group ($P>0.05$) throughout the experiment, whereas P.69 gave some protection at day 3. However P.69 (Fig. 2) provided strong protection against tracheal colonisation in mice compared to control group and the protection was evident throughout the duration of the experiment ($*p<0.05$). There was little difference in *B. pertussis* colonisation of the trachea in the P.30 and control group ($p>0.05$), where the infection reached a peak at day 9 and then showed some clearance by day 14.

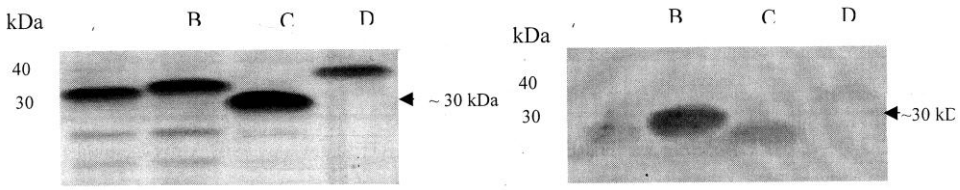

Fig. 1. SDS-PAGE and corresponding immunoblot of the purified recombinant C-terminal domains of the different *B. pertussis* autotransporters including pertactin.
Purified recombinant C-terminal domains subjected to SDS-PAGE (12 %) and stained with coomassie blue and corresponding immunoblot probed with a mouse monoclonal to the PRN C-terminal domain.

A= Purified recombinant C-terminus of Bap-C, B= Purified recombinant C-terminus of PRN, C= Purified recombinant C-terminus of TCF, D= Purified recombinant C-terminus of BrkA

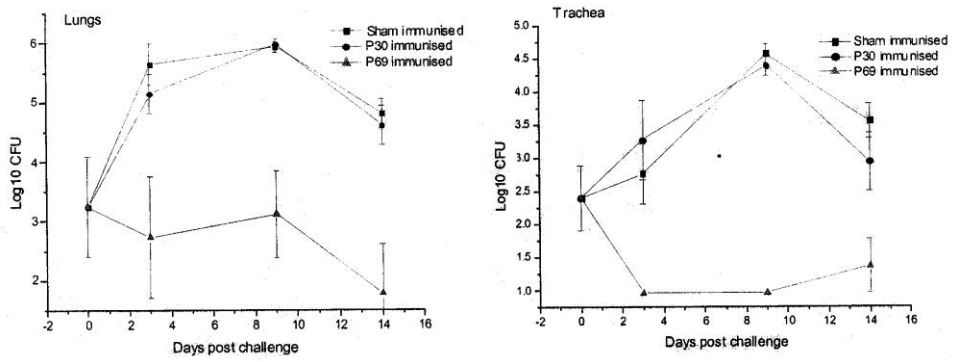

Fig. 2. Mouse protection data showing the CFU recovered from the tracheas and lungs of the mice after intranasal challenge with *B. pertussis* 18-323.

Also (Fig. 2) from the lung counts obtained from the different groups of mice during the course of experiment. It was apparent that immunization with P.69 provided clear protection against colonization of the lungs, as of the trachea. In contrast, the P.30 group did not seem to show any protective effect against the natural course of the *B. pertussis* infection at any stage during the experiment and the lung counts closely paralleled those in the control group, with the infection again reaching a peak around day 9 and thereafter beginning to decline. Mice vaccinated twice subcutaneously with P.69, re-natured P.30 or with the diluent (alhydrogel) only were challenged intranasally with a sub-lethal dose of the standard mouse-virulent *B. pertussis* strain 18-323 (~$1x10^5$ CFU/mouse) and then sacrificed at intervals. One-way analysis of variance (ANOVA) test was applied to determine the statistical difference between the groups. The *P* value <0.0001 is considered extremely significant whereas the *P* value <0.05, is significant. Each point represents the mean of the Log_{10} CFU recovered from the tracheas and lungs of four individual mice and the error bars the S.E.M.

Discussion

Previous studies using the mouse model of infection have suggested that immunisation with purified *B. pertussis* FHA, PTX, Fim or PRN protects against an intranasal or aerosol challenge with *B. pertussis* (Charles *et al*., 1994**;** Mahon and Mills, 1999**;** Shahin and Brodeur, 1994). This is consistent with the presence of antibodies against *B. pertussis* FHA and fimbriae in humans which protect against *B. pertussis* by interfering with its adherence (Shahin and Brodeur, 1994). The antibodies against the other surface structures i.e., LPS (Gerlach *et al*., 2001) and other outer-membrane associated proteins can also reduce the adherence of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* as evident by the protection provided by these immunogens (Hamstra *et al*., (1995).

The protection against *B. pertussis* provided by the members of its autotransporter family is another area to be further exploited by researchers, to enhance the efficacy of the acellular vaccines. Natural and recombinant preparations of purified pertactin/P.69 have been shown to induce a protective immune response against an intranasal or aerosol challenge of *B. pertussis* in experimental models (Boursaux-Eude *et al*., 1999). The protective capacities of purified TCF, BrkA and Vag-8 have not been reported so far. Vag-8 (92 kDa protein) had been reported to be protective only in the presence of non-protective levels of pertussis toxin (Monji *et al*., 1986).

Our previous mouse protection studies using the purified C-terminal domains of *B. pertussis* autotransporter proteins of BrkA, TCF, PRN and Bap-C suggested that they were not immunodominant, protective regions of the autotransporter proteins. These C-terminal domains however, were presented to the mice in denatured form which could be the reason for the lack of provision of protection by the protective epitopes against *B. pertussis*. In the present study, to circumvent the doubt regarding the denatured form of P.30, a re-natured preparation of the recombinant C-terminal domain was used. In parallel, a purified preparation of P.69, which is a known immunogen, was used in its native form.

However, the current study suggested that no protection was afforded by the re-natured P.30 against *B. pertussis* colonisation of the respiratory tract of the mice, whereas P.69 (at the same dose) afforded a clear protection against tracheal and lung colonisation and to some extent colonisation of the nasal cavity. There is a possibility that P.30 may not have an immunodominant epitope which can generate an effective immune response and hence provide protection against *B. pertussis* or, even if it does, there is a possibility that anti-P.30 antibodies may not have access to this antigen because of its presumed embedded nature in the outer membrane (Charles *et al*., 1994). Unfortunately, blood serum samples were not collected from the pre-immunised and post-immunised mice, which would have given useful information regarding the presence of P.30-specific antibodies in the mouse model. On the other hand It has been reported that a good immune response occurs when some *B. pertussis* antigens are presented in their correct form, for example as outer membrane complexes (Gentle *et al.*, 2004; Hamstra *et al*., 1995; Shahin *et al*., 1995). However, the vaccine preparations, challenge doses and routes of administration of the vaccines and adjuvant effects of different formulations are all potential areas to be explored in the field of pertussis vaccinology.

Moreover, the complete genome sequence data is available and it will give information on all the surface exposed and secreted proteins of an organism. This can lead to the identification of potential novel antigens from the genome sequence for possible inclusion in the next generation of acellular vaccines, a strategy known as reverse vaccinology (Preston & Maskell, 2002).

The importance of presenting *B. pertussis* antigens in the correct form has been studied and it appears that the best response occurs in the mouse models when outer membrane complexes or microspheres are used. In this regard Hamstra *et al* (1995) reported that an outer membrane complex (protein-detergent micelles) containing a 32-kDa protein (recently confirmed as the TCF C-terminus) was protective in an intracerebral mouse protection tests. Moreover, the 92 kDa (presumably Vag-8) protein described by Hamstra *et al* (1995) was protective only when non-protective levels of pertussis toxin were present. Monji *et al*., (1986), suggested a role for a 30 kDa protein (possibly the BrkA C-terminus) in *B. pertussis* outer membrane preparations which potentiated an immune response to Haemophilus type B capsular polysaccharide, indicating a role of the C-terminal domains as adjuvants. Similar role for Prn C-terminus should be the subject of research interest in an area of vaccine development.

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