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Abstract

The construction of a hexahistidine-tagged version of the B fragment of diphtheria toxin (DTB) represents an important step in the study of the biological properties of DTB because it will permit the production of pure recombinant DTB (rDTB) in less time and with higher yields than currently available. In the present study, the genomic DNA of the Corynebacterium diphtheriae Park Williams 8 (PW8) vaccine strain was used as a template for PCR amplification of the dtb gene. After amplification, the dtb gene was cloned and expressed in competent Escherichia coli M15™ cells using the expression vector pQE-30™. The lysate obtained from transformed E. coli cells containing the rDTB_PW8 was clarified by centrifugation and purified by affinity chromatography. The homogeneity of the purified rDTB_PW8 was confirmed by immunoblotting using mouse polyclonal anti-diphtheria toxoid antibodies and the immune response induced in animals with rDTB_PW8 was evaluated by ELISA and dermonecrotic neutralization assays. The main result of the present study was an alternative and accessible method for the expression and purification of immunogenically reactive rDTB_PW8 using commercially available systems. Data also provided preliminary evidence that rabbits immunized with rDTB_PW8 are able to mount a neutralizing response against the challenge with toxigenic C. diphtheriae.

Key words: Fragment B; Diphtheria toxin; Diphtheria; dtb gene; E. coli gene expression; Immobilized metal affinity

Introduction

Diphtheria toxin (DT) is an A-B type protein toxin produced by Corynebacterium diphtheriae (1-4). The B fragment (DTB) binds to the receptor on the host cell surface and mediates the translocation of the A fragment (DTA) through the cell membrane, which inactivates the protein synthesis elongation factor 2 in some mammalian cells (5-9). The expression of recombinant DTB (rDTB) in other prokaryotic organisms is necessary to understand the role of DT in the development and severity of toxemic infectious processes (10-12). The expression of rDTB in bacteria was initially considered difficult, due in part to the fact that DTB without DTA was found to be rapidly degraded during the process (13,14). In the early experiments that succeeded in producing rDTB in Escherichia coli, only low yields were achieved (15). Later, Spilsberg et al. (16) constructed a hexahistidine-tagged version of a modified rDTB that was expressed in higher levels by E. coli BL21. Attempts to produce immunogenically reactive rDTB in bacteria in a more accessible form using newer expression systems are of interest. The objective of the present study was to express the dtb gene of the Park Williams 8 (PW8) C. diphtheriae vaccine strain to produce the immunogenically reactive rDTB_PW8 using commercially available expression and purification systems.

Material and Methods

Amplification and cloning of the dtb gene

The pQE30-DTB_PW8 construct was prepared as follows:

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genomic DNA extracted from the C. diptheriae PW8 ATCC 13812 strain was used as a template for PCR amplification of the dtb gene using 5'-GGG ATC CTA GAA GGT AGC TCA TTG -3' as the forward primer and 5'-CCC GGG TGA CCC CAC TAC CTT TCA A -3' as the reverse primer. After purification with the GeneClean® gel extraction kit (BIO 101, USA), the dtb gene was cloned into the expression vector pQE-30™ of the Qiagenexs System based on standard methods described by the manufacturer (Qiagen, USA).

Transformation of E. coli M15™ cells and expression of the dtb gene

The hexahistidine-tagged-fused DTB<sub>PW8</sub> protein was successfully expressed in competent E. coli M15™ cells. During this procedure, E. coli M15™ cells were transformed with the pQE-30/dtb construct and a selected transformant was grown for 12 h at 37°C in 300 mL Luria-Bertani medium containing 25 µg/mL ampicillin to 0.6 absorbance at 600 nm. Subsequently, transfomants were induced with 0.2 mM isopropyl-β-D-thiogalactoside (Promega, USA) for 4 h, collected by centrifugation (10,000 g, 10 min, 4°C), resuspended in 2.0 mL lysis buffer [20 mM Tris-HCl, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (Sigma, USA), 1 µg/mL lysozyme], and incubated for 1 h at 4°C. After 10X 10-s ultrasonic pulses, the suspension was centrifuged (10,000 g, 20 min, 4°C) and the clarified lysate added to a 2-mL suspension of a 50% Superflow Ni-NTA slurry and rotated overnight at 22°C. The mixture was transferred to a 5-mL gravity column and beads were washed twice with 4 mL washing buffer [20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole in phosphate-buffered saline (PBS; Sigma)]. The protein was finally eluted with 4X 0.5 mL elution buffer [20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 8 M urea and 5 mM dithiothreitol (DTT; Sigma)] (16).

SDS-PAGE and densitometric analysis

The material eluted from the Ni-NTA column was analyzed by 12% SDS-PAGE under denaturing conditions (17). Fracitions containing the highest concentration of rDTB<sub>PW8</sub> were dialyzed overnight against 5X PBS containing 0.3 M urea at 4°C. Protein concentration was determined using 200 µL of the recombinant protein rDTB<sub>PW8</sub> in a 100-µL PBS (20%) emulsified in complete Freund’s adjuvant. A booster was given 15 days after immunization. Blood samples were collected from the retro-orbital plexus before immunization and 15 and 60 days thereafter.

A New Zealand rabbit was immunized intradermally in the thigh with 2 µg rDTB<sub>PW8</sub> in 0.1-mL PBS supplemented with complete Freund’s adjuvant and boosted with the same formulation 21 days later. Blood samples were collected from saphena or ear veins before challenge at 21 and 28 days thereafter.

Western blotting and localization of the heterologous protein in E. coli M15™ cells

Western blotting analysis of rDTB<sub>PW8</sub> fractions was performed using standard procedures (20). Proteins were blotted onto a 0.45-µm nitrocellulose membrane and blocked overnight with 5% skim milk/PBS/0.1% Tween 20 (PBS-T). On the next day, the blocked membrane was incubated with a 1:1000 mouse polyclonal antibody against the antitoxin toxoid produced in house and then with alkaline phosphatase-conjugated anti-mouse IgG (Sigma) diluted in PBS-T. Blots were further developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Sigma).

Immunization of mice and rabbits

Immunization experiments with rDTB<sub>PW8</sub> were conducted on 4- to 6-week-old male BALB/c mice and New Zealand rabbits in compliance with the Ethical Principles in Animal Experimentation established by the Brazilian College of Animal Experimentation and approved by the Fundação Instituto Oswaldo Cruz - Animal Use Ethics Committee - CEUA (P0163-03) under protocol #CEUA L00034-07.

Mice (N = 5) were immunized intraperitoneally with 20 µg of the recombinant protein rDTB<sub>PW8</sub> in a 100-µL PBS (20%) emulsified in complete Freund’s adjuvant. A booster was given 15 days after immunization. Blood samples were collected from the retro-orbital plexus before immunization and 15 and 60 days thereafter.

A New Zealand rabbit was immunized intradermally in the thigh with 2 µg rDTB<sub>PW8</sub> in 0.1-mL PBS supplemented with complete Freund’s adjuvant and boosted with the same formulation 21 days later. Blood samples were collected from saphena or ear veins before challenge at 21 and 28 days thereafter.

Detection of rDTB<sub>PW8</sub>-specific antibodies by ELISA

ELISA was performed for the detection and quantification of both anti-rDTB<sub>PW8</sub> mouse and rabbit antibodies (21). Wells of Maxisorp plates were coated with 0.1 µg DT (Sigma) in 100 µL PBS. After overnight incubation at 4°C, microplates were washed with PBS-T. 100 µL goat anti-rabbit IgG or goat anti-mouse IgG (1:4000) conjugated with horseradish peroxidase (both from Sigma) was added to each well and the plates were incubated for 1 h at 37°C. After washing, reactions were observed 10 min after incubation at room temperature and in the absence of light with 10 mg/mL 3, 3',5,5' tetramethyl benzidine in 100 µL citrate phosphate buffer and 0.01% hydrogen peroxide as substrate. Finally, the reaction was stopped by the addition of 50 µL 2 N sulfuric acid, and the absorbance at 450 nm of the yellow-orange color was measured with a spectrophotometer.

DT dermonecrotic neutralization test in rabbits

In order to evaluate the in vivo dermonecrotic neutralization potential of the recombinant DTB<sub>PW8</sub> protein
we used an adaptation of Fraser’s protocol (22). Seven days after the booster dose with DTBPW8, an animal was challenged on the shaved back with injections of 0.1 mL bacterial supernatants adjusted to a concentration of approximately 3 x 10^8 CFU toxigenic PW8 vaccine strain and nontoxigenic ATCC 27010 type strain. The efficacy of DT neutralization was monitored visually and sized when possible at the site of injection after 24, 48, and 120 h, for the absence or presence of dermoreactions to the bacterial challenges. Similarly, a non-immunized rabbit was also challenged on the shaved back with the supernatants of both bacterial strains.

**Results**

**Amplification of the dtb gene of the *C. diphtheriae* PW8 vaccine strain by PCR**

Following the purification step with the Gene-Clean Extraction kit the 1051-bp amplicon was cloned in frame within the BamHI and SmaI restriction sites of the pQE-30™ expression vector to create a fusion product with the hexahistidine tag-coding sequence as shown in Figure 1A. Competent *E. coli* M15™ cells were successfully transformed with the ligation product and the details of the plasmid and the inserted DTB are presented in Figure 1B and C.

**Expression of the rDTBPW8 recombinant protein**

Expression of rDTBPW8 recombinant protein in *E. coli* M15™ cells was demonstrated by SDS-PAGE and immunoblot analysis (Figure 2). The clarified rDTBPW8 lysate was purified in one step using nickel-coated agarose beads and the eluted material was submitted to analysis by SDS-PAGE. A final protein concentration of 0.38 mg/mL was observed in the pool of rDTBPW8-containing fractions. rDTBPW8 (lane 7) was recognized by anti-DT antibodies in Western blotting assays, confirming that the purified product was a fragment of DT protein.

**Densitometric analysis**

SDS-PAGE of the crude rDTBPW8 preparation revealed various polypeptide bands suggestive of minor polypeptide contaminants. Densitometric analysis of these bands showed the relative abundance of each polypeptide as well as their estimated molecular weight (Figure 3A and B). As expected, the highest peak (84.8%) corresponded to the fraction containing the target protein rDTBPW8. On the other hand, we showed that Superdex-200 chromatography of the same preparation can be used prior to affinity chromatography to improve the fractionation in immobilized metal ion affinity chromatography columns and to improve the monitoring of the progress of the protein in the chromatograms. Such progress was indicated by the increase in intensity of the peptide peaks. The highest peak corresponded to the fraction containing the target protein rDTBPW8 according to SDS-PAGE analysis (Figure 3C).

**Humoral immune response of mice and rabbits immunized with rDTBPW8**

The results presented in Figure 4 indicate significant differences between the antigenic and immunogenic prop...
erties of rDTB<sub>PW8</sub> in mice (Figure 4A) and rabbits (Figure 4B). Both types of animal responded to the purified protein. However, while an antibody response of approximately 20 ELISA units/mL was observed in mice 60 days after immunization, a higher response (150 ELISA units/mL) was observed in rabbits starting at day 21 post-immunization.

Figure 3. Densitometric analysis of recombinant diphtheria toxin Park Williams 8 (rDTB<sub>PW8</sub>) protein bands. A, SDS-PAGE gel stained with Coomassie brilliant blue R-350. MW = molecular weight markers. B, Table displaying the relative abundance of polypeptide-stained bands and their estimated molecular mass. C, Crude preparation of diphtheric protein subunit B chromatographed onto Superdex-200. The highest peak with a molecular mass near 40 kDa corresponds to the fraction containing the target protein (rDTB<sub>PW8</sub>).

Figure 4. Antibody response of mice (N = 5) (A) and of a rabbit (N = 1) (B) after immunization with recombinant diphtheria toxin Park Williams 8 (rDTB<sub>PW8</sub>). Circulating IgG was measured by ELISA units/mL (EU/mL).
The IgG response to the rDTB
PW8
protein was much stronger in the rabbit than in mice.

**DT demonecrotic neutralization test in rabbits immunized with rDTB
PW8**

The reading for the non-vaccinated animal at the site challenged with the toxigenic PW8 strain revealed a local of necrosis at 24 h, which increased up to 1 cm in diameter at 120 h, as illustrated in Figure 5A. On the other hand, the rabbit vaccinated with rDTB
PW8
(Figure 5B) remained unharmed after 120 h and presented no more than a slight induration at the site of the challenge with the virulent strain. As expected, no reactions were detected at the sites of injection with the non-virulent strain in either animal species. Animals were sacrificed 1 week after the challenge and no evidence of bacterial dissemination in their organs was detected.

**Discussion**

In the present study, genomic DNA from the *C. diphtheriae* PW8 vaccine strain was used as a template for PCR amplification of the *dtb* gene. The amplicon obtained was sequenced and found to contain the entire B fragment DNA sequence from nucleotide 888 to 1939, as reported previously (23).

rDTB has been produced by different laboratories in the last few years (16,24). While Johnson et al. (24) used the pGEMEX expression plasmid from Promega™, Spilsberg et al. (16) used the pET21d+ vector from Novagen™. However, neither group studied the neutralization potency of the immune response induced by DTB. In our study, we produced the DTB fragment using the pQE-30 expression vector from Quiagen™. The major feature of this system is the expression of the recombinant protein fused to a hexahistidine tag, which is important for purification. In the present study, despite the high level of expression in *E. coli* M15™ the yield of the purified rDTB
PW8
was low (0.38 mg/mL) because most of the protein was insoluble (>70%). However, this yield can be improved by optimization of the production and purification protocols.

The densitometric analysis of the bands separated by electrophoresis of the crude rDTB
PW8
preparation showed, as expected, that the highest peak (84.8%) contained the target protein rDTB
PW8
. We also demonstrated for the same preparation that Superdex-200 chromatography can be used prior to affinity chromatography to improve the resolution of the target-protein in immobilized metal ion affinity chromatograph columns. Such progress was indicated by the increase in intensity of the peptide peaks.

The humoral immune response against rDTB
PW8
was evaluated in mice and rabbits by ELISA. Both species of animals responded to immunization with the DTB fragment; however, rabbits mounted a much stronger IgG antibody response than mice. In fact, it should be pointed out that in our study rabbits were immunized with ten times less antigen than mice. These results suggest that mice are less sensitive to the DT than rabbits. For this reason, we decided to evaluate the neutralization capacity of rDTB
PW8
in the rabbit model.

The first *in vivo* assay for the determination of the virulence of diphteria bacilli was developed by Fraser (22) in 1931. The test is very sensitive and is based on the estimation of antitoxin levels on the skin of rabbits immunized or not with the antiphthetic vaccine and on the demonstration of the presence/absence of typical reactions at the site of injection of the bacterial challenge. Fraser’s original assay was performed in rabbits and was recommended whenever results from the guinea pigs and Elek tests were negative (23). We also used rabbits to assay the
protective effect (neutralization) of rDTB<sub>PW8</sub> because, in addition to their ability to mount a superior IgG response after immunization with the purified B fragment, rabbits are much larger animals and therefore are more appropriate for the skin demonecrotic neutralization test designed by Fraser. Our results demonstrated that the rabbit immunized with rDTB<sub>PW8</sub> was capable of mounting an effective neutralization response against the virulent challenge of the PW8 strain of <i>C. diphtheriae</i>, as opposed to the non-immunized animal and, consequently, that rDTB<sub>PW8</sub> is able to induce a potent neutralizing response against DT in immunized rabbits.

The present study has provided additional evidence about an alternative and accessible method for the expression and purification of the immunogenically reactive fragment B (rDTB<sub>PW8</sub>) of diphtheria toxin from the <i>C. diphtheriae</i> PW8 vaccine strain using a commercially available expression system. More importantly, we also provide preliminary data about the protective potential of the DTB fragment against the challenge with toxigenic corynebacteria in rabbits.

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**References**
