BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH

www.bjournal.com.br

ISSN 0100-879X
Volume 43 (5) 409-521 May 2010

BIOMEDICAL SCIENCES

Braz J Med Biol Res, May 2010, Volume 43(5) 460-466

Expression and purification of the immunogenically active fragment B of the Park Williams 8 Corynebacterium diphtheriae strain toxin

D.V. Nascimento, E.M.B. Lemes, J.L.S. Queiroz, J.G. Silva Jr., H.J. Nascimento, E.D. Silva, R. Hirata Jr., A.A.S.O. Dias, C.S. Santos, G.M.B. Pereira, A.L. Mattos-Guaraldi and G.R.G. Armoa

Brazilian Journal of Medical and Biological Research (2010) 43: 460-466
ISSN 0100-879X

Expression and purification of the immunogenically active fragment B of the Park Williams 8 *Corynebacterium diphtheriae* strain toxin

D.V. Nascimento[1,4], E.M.B. Lemes[1], J.L.S. Queiroz[1], J.G. Silva Jr.[1], H.J. Nascimento[1], E.D. Silva[1], R. Hirata Jr.[4], A.A.S.O. Dias[3], C.S. Santos[4], G.M.B. Pereira[2,4], A.L. Mattos-Guaraldi[4] and G.R.G. Armoa[2]

[1]Instituto de Tecnologia em Imunobiológicos, [2]Instituto Oswaldo Cruz, [3]Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil
[4]Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

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:

Correspondence: A.L. Mattos-Guaraldi, Faculdade de Ciências Médicas, UERJ, Av. 28 de Setembro, 87 - Fundos, 3º andar, 20551-030 Rio de Janeiro, RJ, Brasil. Fax: +55-21-2587-6476. E-mail: guaraldi@pq.cnpq.br; guaraldi@uerj.br

Received September 29, 2009. Accepted April 6, 2010. Available online April 22, 2010. Published May 14, 2010.

genomic DNA extracted from the *C. diphtheriae* 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 G -3' as the reverse primer. After purification with the Gene-Clean® gel extraction kit (BIO 101, USA), the *dtb* gene was cloned into the expression vector pQE-30™ of the QIAexpress 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_{PW8} 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, transformants 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). Fractions containing the highest concentration of $rDTB_{PW8}$ were dialyzed overnight against 5X PBS containing 0.3 M urea at 4°C. Protein concentration was determined using a BioRad Protein Assay™ Kit (USA), based on the method of Bradford, and densitometric analysis was performed with an Image Master 1-D densitometer (GE Healthcare, USA) as follows. The crude $rDTB_{PW8}$ preparation was chromatographed through a previously equilibrated affinity column containing a nickel streamline chelating matrix. Elution was performed using 20 mM Tris-HCl buffer containing 8 M urea, 0.5 M NaCl, 5 mM DTT and 0.5 M imidazole. The affinity chromatography fractions were analyzed by 12% SDS-PAGE. The crude $rDTB_{PW8}$ preparation was also submitted to sieving exclusion chromatography on a Superdex 200 column (Pharmacia, USA) previously equilibrated with 50 mM Tris-HCl. Effluent absorbing at 220 nm was combined and analyzed by SDS-PAGE as described (18,19).

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

Western blotting analysis of $rDTB_{PW8}$ 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 1:1000 mouse polyclonal antibody against the antidiphtheria 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 $rDTB_{PW8}$ 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 $rDTB_{PW8}$ 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 $rDTB_{PW8}$ 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 $rDTB_{PW8}$-specific antibodies by ELISA

ELISA was performed for the detection and quantification of both anti-$rDTB_{PW8}$ 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_{PW8} protein

we used an adaptation of Fraser's protocol (22). Seven days after the booster dose with DTB_{PW8}, 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 *Bam*HI and *Sma*I 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 $rDTB_{PW8}$ recombinant protein

Expression of $rDTB_{PW8}$ recombinant protein in *E. coli* M15™ cells was demonstrated by SDS-PAGE and immunoblot analysis (Figure 2). The clarified $rDTB_{PW8}$ 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 $rDTB_{PW8}$-containing fractions. $rDTB_{PW8}$ (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 $rDTB_{PW8}$ 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 $rDTB_{PW8}$. 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 $rDTB_{PW8}$ according to SDS-PAGE analysis (Figure 3C).

Humoral immune response of mice and rabbits immunized with $rDTB_{PW8}$

The results presented in Figure 4 indicate significant differences between the antigenic and immunogenic prop-

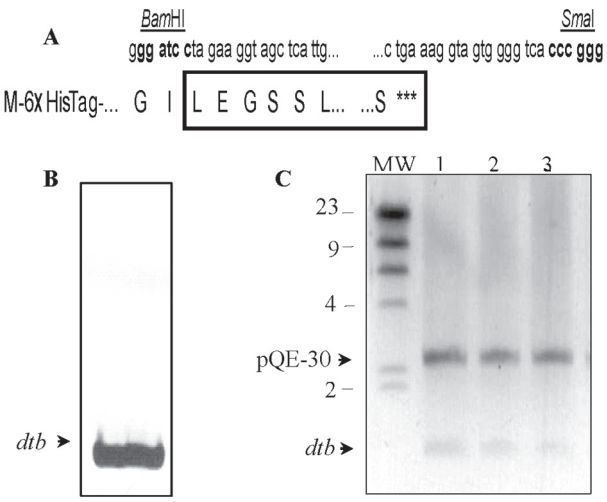


Figure 1. Amplification and cloning of the *Corynebacterium diphtheriae* Park Williams 8 (PW8) 1051-bp *dtb* gene. *A*, Restriction sites are indicated in bold and the translated amino acid sequences from the N-terminal to the C-terminal end are shown in upper case in a one-letter code; the DTB_{PW8} amino acid sequence is boxed and the hexahistidine tag (HisTag) is shown as 6X HisTag. *B*, *dtb* gene from *C. diphtheriae* PW8 vaccine strain amplified by PCR. *C*, Agarose gel electrophoresis (0.8%) of three pQE-30 DTB_{PW8} clones after digestion with *Bam*HI and *Sma*I. MW = molecular weight markers.

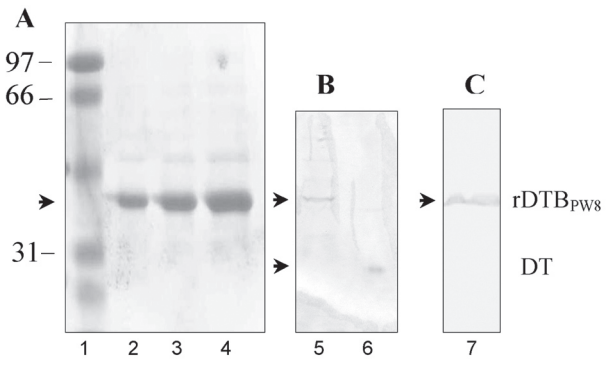
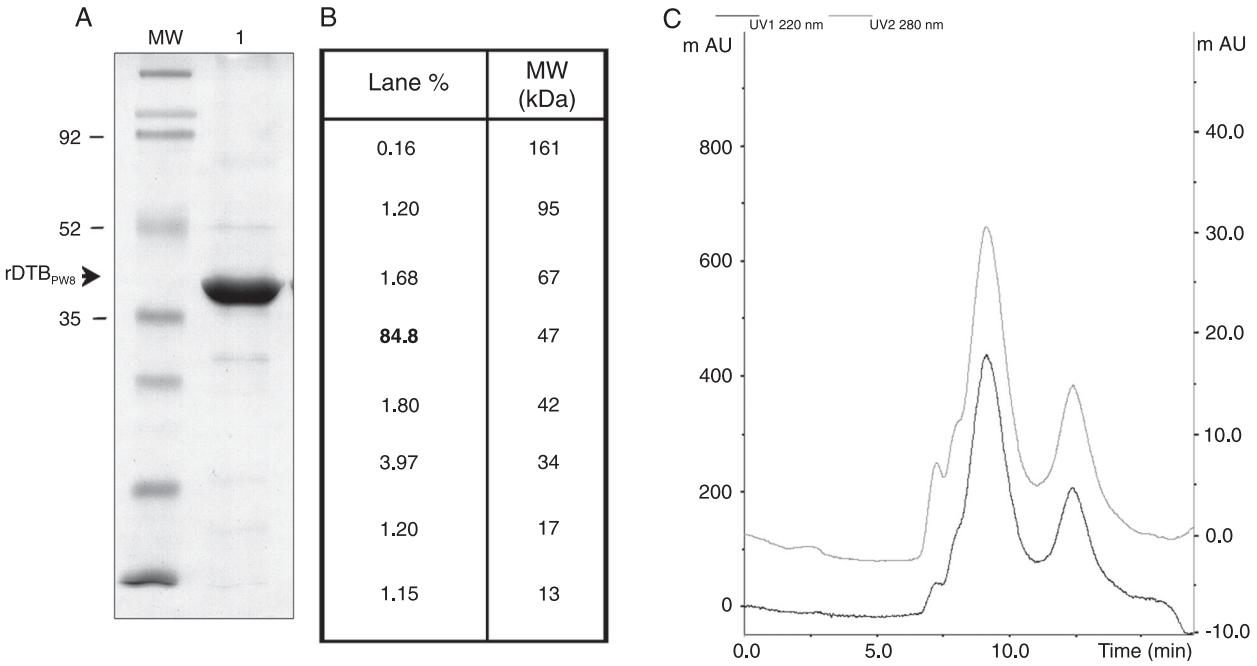


Figure 2. Expression of the B fragment of recombinant diphtheria toxin Park Williams 8 ($rDTB_{PW8}$) in *Escherichia coli* M15™ cells. *A*, SDS-PAGE (12%) of fractions obtained during purification with Ni-NTA Superflow. Polypeptide bands were stained with the Coomassie brilliant blue R-350 reagent: *Lane 1*, MW = molecular weight markers; *lane 2*, flow-through fraction; *lanes 3* and *4*, wash fractions. *B*, *Lane 5*, eluted and dialyzed fraction; *lane 6*, diphtheria toxin (positive-control). *C*, Immunoblotting using mouse polyclonal antibody against the antidiphtheria toxoid produced in house (lane 7).

erties of $rDTB_{PW8}$ 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.



Lane %	MW (kDa)
0.16	161
1.20	95
1.68	67
84.8	47
1.80	42
3.97	34
1.20	17
1.15	13

Figure 3. Densitometric analysis of recombinant diphtheria toxin Park Williams 8 ($rDTB_{PW8}$) 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 ($rDTB_{PW8}$).

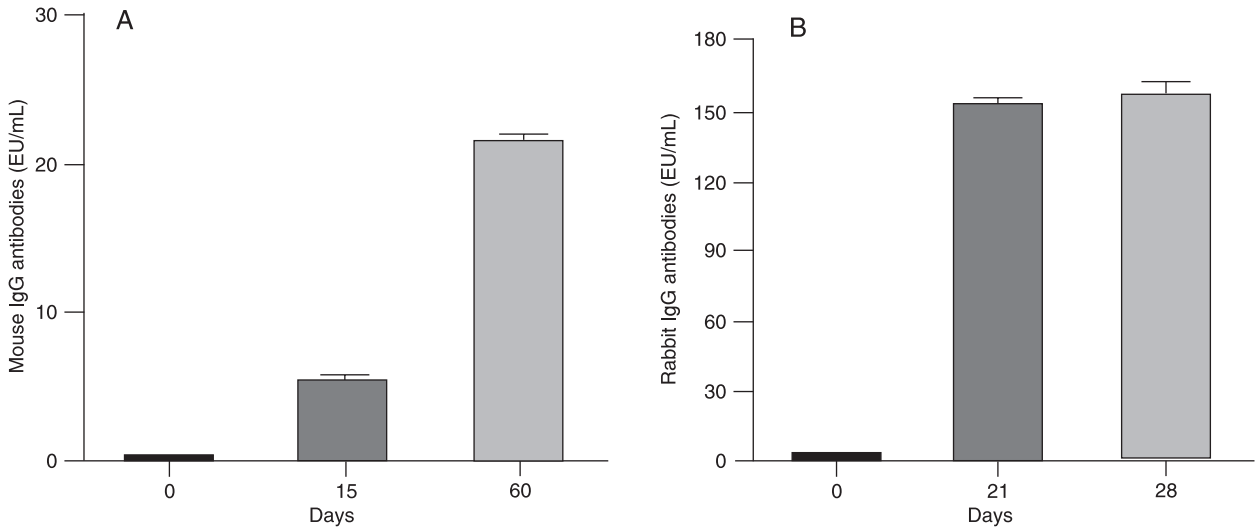


Figure 4. Antibody response of mice (N = 5) (A) and of a rabbit (N = 1) (B) after immunization with recombinant diphtheria toxin Park Williams 8 ($rDTB_{PW8}$). Circulating IgG was measured by ELISA units/mL (EU/mL).

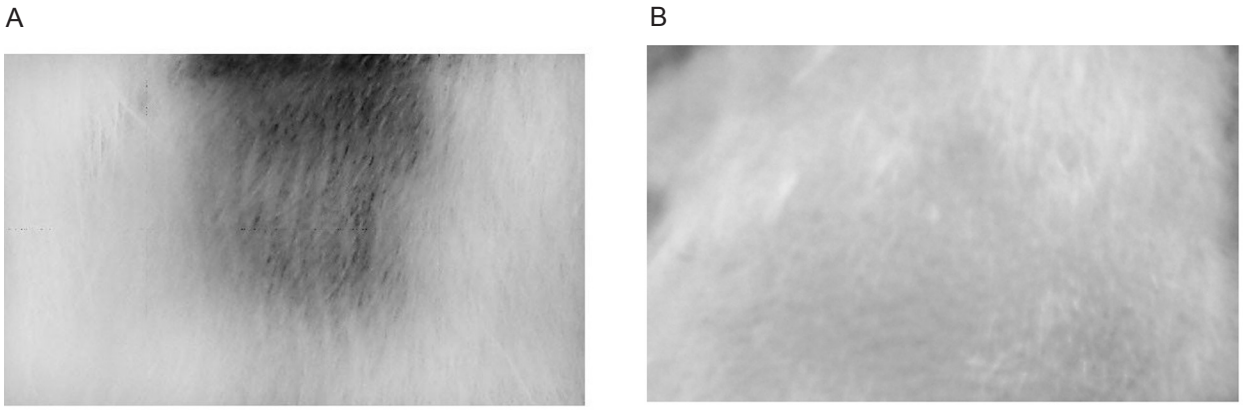


Figure 5. DT dermonecrotic neutralization test in rabbits immunized with $rDTB_{PW8}$. *A*, At 120 h post-challenge with the supernatant of the toxigenic PW8 strain (approximately 3 x 10^8 CFU), the readings over the shaved back of the non-vaccinated animal revealed an area of necrosis (up to 1 cm in diameter). *B*, The vaccinated animal remained unharmed and presented no more than a slight induration at the site of challenge.

The IgG response to the $rDTB_{PW8}$ protein was much stronger in the rabbit than in mice.

DT dermonecrotic 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 diphtheria 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 antidiphtheritic 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 $rDTB_{PW8}$ 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 dermonecrotic neutralization test designed by Fraser. Our results demonstrated that the rabbit immunized with $rDTB_{PW8}$ was capable of mounting an effective neutralization response against the virulent challenge of the PW8 strain of *C. diphtheriae*, as opposed to the non-immunized animal and, consequently, that $rDTB_{PW8}$ 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 ($rDTB_{PW8}$) of diphtheria toxin from the *C. diphtheriae* 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.

Acknowledgments

This study was carried out in partial fulfillment of the requirements of a PhD thesis for D.V. Nascimento, Faculdade de Ciências Médicas (PGCM), Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brazil. Research supported by Bio-Manguinhos/FIOCRUZ, PAPES II/FIOCRUZ, FAPERJ, CNPq, CAPES, Programa de Núcleo de Excelência (PRONEX/MCT/CNPq), and Programa de Pós-Doutorado-PAPD (FAPERJ/CAPES).

References

1. Pappenheimer AM Jr. Diphtheria toxin. *Annu Rev Biochem* 1977; 46: 69-94.
2. Mattos-Guaraldi AL, Moreira LO, Damasco PV, Hirata Junior R. Diphtheria remains a threat to health in the developing world - an overview. *Mem Inst Oswaldo Cruz* 2003; 98: 987-993.
3. Hansmeier N, Chao TC, Kalinowski J, Puhler A, Tauch A. Mapping and comprehensive analysis of the extracellular and cell surface proteome of the human pathogen *Corynebacterium diphtheriae*. *Proteomics* 2006; 6: 2465-2476.
4. Collier RJ. Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. *Toxicon* 2001; 39: 1793-1803.
5. Collier RJ. Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. *J Mol Biol* 1967; 25: 83-98.
6. Sandvig K, Olsnes S. Rapid entry of nicked diphtheria toxin into cells at low pH. Characterization of the entry process and effects of low pH on the toxin molecule. *J Biol Chem* 1981; 256: 9068-9076.
7. Sandvig K, Olsnes S. Diphtheria toxin-induced channels in Vero cells selective for monovalent cations. *J Biol Chem* 1988; 263: 12352-12359.
8. Stenmark H, Olsnes S, Sandvig K. Requirement of specific receptors for efficient translocation of diphtheria toxin A fragment across the plasma membrane. *J Biol Chem* 1988; 263: 13449-13455.
9. Stenmark H, Afanasiev BN, Ariansen S, Olsnes S. Association between diphtheria toxin A- and B-fragment and their fusion proteins. *Biochem J* 1992; 281 (Part 3): 619-625.
10. Zanen J, Muyldermans G, Beugnier N. Competitive antagonists of the action of diphtheria toxin in HeLa cells. *FEBS Lett* 1976; 66: 261-263.
11. Cabiaux V, Brasseur R, Wattiez R, Falmagne P, Ruysschaert JM, Goormaghtigh E. Secondary structure of diphtheria toxin and its fragments interacting with acidic liposomes studied by polarized infrared spectroscopy. *J Biol Chem* 1989; 264: 4928-4938.
12. Spilsberg B, Hanada K, Sandvig K. Diphtheria toxin translocation across cellular membranes is regulated by sphingolipids. *Biochem Biophys Res Commun* 2005; 329: 465-473.
13. Yamaizumi M, Uchida T, Takamatsu K, Okada Y. Intracellular stability of diphtheria toxin fragment A in the presence and absence of anti-fragment A antibody. *Proc Natl Acad Sci U S A* 1982; 79: 461-465.
14. Papini E, Rappuoli R, Murgia M, Montecucco C. Cell penetration of diphtheria toxin. Reduction of the interchain disulfide bridge is the rate-limiting step of translocation in the cytosol. *J Biol Chem* 1993; 268: 1567-1574.
15. Cabiaux V, Phalipon A, Wattiez R, Falmagne P, Ruysschaert JM, Kaczorek M. Expression of a biologically active diphtheria toxin fragment B in *Escherichia coli*. *Mol Microbiol* 1988; 2: 339-346.
16. Spilsberg B, Sandvig K, Walchli S. Reconstitution of active diphtheria toxin based on a hexahistidine tagged version of the B-fragment produced to high yields in bacteria. *Toxicon* 2005; 46: 900-906.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.
18. Wang H, Del Grosso AV, May JC. Determination of benzethonium chloride in anthrax vaccine adsorbed by HPLC. *Biologicals* 2006; 34: 257-263.
19. Azarkan M, Huet J, Baeyens-Volant D, Looze Y, Vandenbussche G. Affinity chromatography: a useful tool in proteomics studies. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 849: 81-90.
20. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979; 76: 4350-4354.
21. Gor DO, Ding X, Li Q, Greenspan NS. Genetic fusion of three tandem copies of murine C3d sequences to diphtheria toxin fragment B elicits a decreased fragment B-specific antibody response. *Immunol Lett* 2006; 102: 38-49.
22. Fraser DT. The technique of a method for the quantitative determination of diphtheria antitoxin by a skin test in rabbits. *Transact Royal Soc Canada* 1931; 5: 175-181.

23. Ratti G, Rappuoli R, Giannini G. The complete nucleotide sequence of the gene coding for diphtheria toxin in the *Corynephage omega* (tox+) genome. *Nucleic Acids Res* 1983; 11: 6589-6595.

24. Johnson N, Pickett MA, Watt PJ, Clarke IN, Heckels JE. Construction of an epitope vector utilising the diphtheria toxin B-subunit. *FEMS Microbiol Lett* 1997; 146: 91-96.