Vaccine 29 (2011) 5978–5985

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Co-expression of tetanus toxin fragment C in *Escherichia coli* with thioredoxin and its evaluation as an effective subunit vaccine candidate

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ARTICLE INFO

Article history:
Received 11 March 2011
Received in revised form 2 May 2011
Accepted 13 June 2011
Available online 28 June 2011

Keywords:
Clostridium tetani
Tetanus toxin
Fragment C
Ganglioside
Vaccine

ABSTRACT

The receptor-binding domain of tetanus toxin (THc), which mediates the binding of the toxin to the nerve cells, is a candidate subunit vaccine against tetanus. In this study one synthetic gene encoding the THc was constructed and highly expressed in *Escherichia coli* by co-expression with thioredoxin (Trx). The purified THc-vaccinated mice were completely protected against an active toxin challenge in mouse models of disease and the potency of two doses of THc was comparable to that of three doses of toxoid vaccine. And a solid-phase assay showed that the anti-THc sera inhibited the binding of THc or toxoid to the ganglioside GT1b as the anti-tetanus toxoid sera. Furthermore, mice were vaccinated once or twice at four different dosages of THc and a dose–response was observed in both the antibody titer and protective efficacy with increasing dosage of THc and number of vaccinations. The data presented in the report showed that the recombinant THc expressed in *E. coli* is efficacious in protecting mice against challenge with tetanus toxin suggesting that the THc protein may be developed into a human subunit vaccine candidate designed for the prevention of tetanus.

1. Introduction

Tetanus toxin (TeNT) is very toxic and the estimated human lethal dose is less than 2.5 ng per kg. TeNT is a potent neurotoxin synthesized by *Clostridium tetani*. TeNT is a 150 kDa protein composed of three domains, each of approximately 50 kDa, e.g., an N-terminal catalytic domain (light chain), an internal heavy chain translocation domain (HN domain) and the C-terminal heavy chain receptor-binding domain (THc domain) [1–4]. Current tetanus vaccines are produced by formaldehyde inactivation of tetanus toxin (toxoid) isolated from cultures of toxin-producing *C. tetani*, which is a time-consuming and technically demanding procedure. And these toxoid vaccine preparations are only partially pure and occasionally give rise to adverse reactions on hyper-immunization [5–7]. A recombinant subunit vaccine against tetanus would eliminate the need to culture *C. tetani* or purify tetanus toxin. For example, fragment C (THc), the 50-kDa carboxy-terminal portion of tetanus toxin, which expressed and purified from *Escherichia coli* and yeast cells [8–16], is nontoxic but has ganglioside [15–20] binding activities, and can induce protective immune responses against tetanus toxin following parenteral immunization. DNA vaccines encoding the THc domain have been described as alternate candidate [21,22]. In addition, oral or intranasal vaccines against tetanus toxin based on bacterial vectors or edible plant vaccines expressing fragment C are under development [23–27].

Except that the nontoxic fragment C of tetanus toxin has been used as a potential subunit candidate vaccine, recombinant tetanus toxin fragment C as vaccine carrier could dramatically enhance immunogenicity and protective efficacy of conjugate or fusion vaccines [28–31]. Furthermore, fragment C of tetanus toxin, more than a carrier, also was a potential vector for delivering heterologous proteins to neurons in non-viral gene therapy [32–36]. Therefore, recombinant tetanus toxin fragment C as vaccine antigen or vectors for conjugate vaccines has been also explored in our laboratory. And we have previously reported that soluble and stable recombinant Hc domains of botulinum neurotoxin serotypes A and F was highly expressed and purified from *E. coli* and the efficacy of these Hc subunit vaccines against biologically active BoNTs was demonstrated [37–39]. First, in the current study one synthetic gene encoding the nontoxic 50 kDa C-terminal domain of TeNT (THc) was constructed and highly expressed in *E. coli* by co-expression with thioredoxin (Trx). Further, we compare the properties of THc with tetanus toxoid on ganglioside binding activities and assess their

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doi:10.1016/j.vaccine.2011.06.039

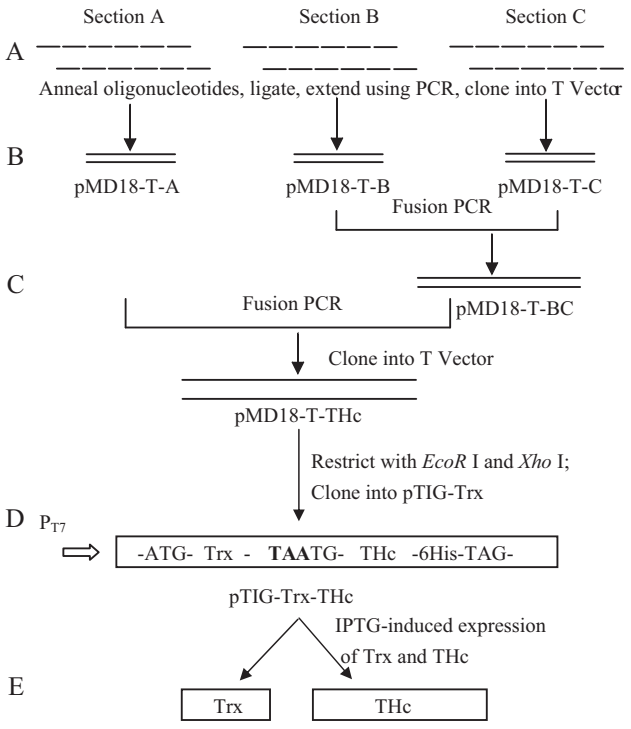

Fig. 1. Construction, cloning and expression of THc in *E. coli*. A. The synthetic *THc* gene was constructed from three blocks, each of which was constructed from 12 overlapping oligonucleotides which were annealed, ligated and made double stranded using PCR. B. Blocks A–C were subcloned into plasmid pMD18-T (T Vector) and the nucleotide sequence of the three blocks was sequenced. C. After the about 870 bp BC block was constructed by fusion PCR, the synthetic *THc* gene was constructed by fusion PCR and subcloned into pMD18-T, the nucleotide sequence of complete *THc* was sequenced. D. The *THc* coding region was digested with *EcoR* I and *Xho* I and subcloned into pTIG-Trx; core sequence of the encoding region: 5′-TAAGGAG (ribosome binding site, RBS) GAGATATACATATG (*Nde* I, ATG is the first AUG start codon)-*Trx*-GCGGGATCCGGTAAGGAG (RBS) GAATTC (*EcoR* I) TAATG (TAA, the first stop codon for *Trx*, and ATG, the second AUG start codon for *THc*, share together one A)-*THc*-CTCGAG (*Xho* I)-*6His*-TAG (the second stop codon for *THc*)-3′. E. The soluble THc was expressed in *E. coli* (BL21) and the Trx protein was also co-expressed.

immunogenicities and protective capacities against TeNT challenge in mice. The data presented in this report demonstrate that the recombinant THc protein may be developed into a novel and highly effective human subunit vaccine candidate designed for the prevention of tetanus or be used as a potential vector for the development of conjugate vaccines and immunological therapeutics.

2. Materials and methods

2.1. Construction of the THc gene

Initially, construction of the synthetic *THc* gene encoding the nontoxic 50 kDa C-terminal domain of TeNT (THc, amino acids 884–1 315, ~50 kDa) was divided into three sections (Fig. 1A), A (N terminal end of THc) to C (C terminal end of THc). In brief, each section (about 448 bp in length) was constructed with 12 overlapping oligonucleotides which were synthesized by Invitrogen Biotechnology Co., Ltd (Shanghai, China). These oligonucleotides were typically about 72 bases in length and contained complementary sequences (15 bases) at the 5′ and 3′ ends to allow annealing (Fig. 1A). These sections were made double stranded and amplified using the polymerase chain reaction (PCR) with LA Taq polymerase (TaKaRa) and further subcloned into pMD18-T (TaKaRa) in *E. coli* strain DH5α, respectively (Fig. 1B). Following sequencing, sections B and C were fused into BC by fusion PCR. Following the same methods, ABC, containing the complete *THc* gene of 1 296 bp, was obtained and subcloned into pMD18-T (Fig. 1C). The correct clone with the *THc* gene, confirmed by sequencing, was named pMD18-T-THc.

2.2. Expression of THc in E. coli and its purification

The pMD18-T-THc DNA plasmid containing the complete *THc* gene was digested with *EcoR* I and *Xho* I to excise the *THc* DNA fragment, which then was subcloned into an expression vector pTIG-Trx plasmid digested by the same enzymes to create recombinant plasmid pTIG-Trx–THc as described previously (Fig. 1D) [37,39]. The correct clone pTIG-Trx–THc was transformed into *E. coli* strain BL21 (DE3) cells (Stratagene) and cultures of the recombinant BL21 were grown in Luria Bertani (LB) containing 100 μg/ml ampicillin at 37 °C until an optical density at 600 nm was 0.5. Isopropyl-B-D-thiogalactopyranoside (1 M, IPTG, Sigma) was added to the culture at a final concentration of 0.4 mM and growth was continued at 250 rpm for 8–12 h at 16 °C (Fig. 1E). The cells were grown in 1000 ml of LB and harvested cells were resuspensed in buffer A (20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl), and lysed by sonication. The resulting lysates were centrifuged at 15,000 × *g* for 30 min at 4 °C and the six His-tag fusion proteins were purified by nickel affinity column chromatography (Amersham Biosciences) according to the recommendation of the manufacturer. In brief, the lysates were loaded on a column of Ni-NTA resin (5 ml bed volume) that had been equilibrated with 25 ml buffer A. The column was washed with 40 ml buffer A followed by 20 ml buffer B (buffer A containing 20 mM imidazole) and then eluted with 10 quantities of 1 ml buffer C (buffer A containing 500 mM imidazole). The fraction contained the THc product was dialyzed at 4 °C against 20 vol of 20 mM sodium acetate, pH 4.5 to decrease the sample conductivity in preparation for the second chromatography step.

Then, HiTrapTM SP FF (5 ml) (Pharmacia Biotech, Sweden) was used in the second purification step by the basic charge of THc (p*I* 6.6). The column was equilibrated with 10 column volumes (CV) of acetate buffer (20 mM sodium acetate, pH 4.5) at a rate of 100 cm/h and then loaded with the product from the first step at a rate of 60 cm/h and then washed with 10 CV of equilibration buffer containing 50–1000 mM NaCl at a rate of 100 cm/h. Recombinant product was eluted from the column using step elution with 6 CV of 20 mM Tris–HCl (pH 8.5) and 20 mM Tris–HCl containing 1000 mM NaCl at a rate of 60 cm/h. The fraction THc obtained from the SP column was dialyzed at 4 °C against 20 vol of 20 mM sodium phosphate buffer (pH 8.0) and frozen at −70 °C or −20 °C for storage. Protein concentrations were estimated by using BCA protein assay (Sigma) according to the manufacturer's protocol.

The soluble fraction and the purified THc were verified by 12% SDS-PAGE and Western blot using hyperimmune mouse tetanus toxin antiserum. After separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond-C, Amersham) with semi-dry blot apparatus (Semi-dry Transfer Cell, Bio-RAD) and blocked with 5% nonfat dry milk for 2 h. The blots were subsequently incubated with 1:200 dilution of mouse anti-TeNT serum for 1 h at room temperature. After washing the blots were then incubated with 1:2000 dilution of goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc.), washed and 5 ml of DAB substrate were added to visualize binding.

2.3. Vaccination of mice and challenge with tetanus toxin

Specific pathogen-free female BALB/c mice (purchased from Beijing Laboratory Animal Center, Beijing) 6 weeks of age were randomly assigned to different treatment groups. In the first vaccination study, groups of eight mice were vaccinated with 1 or 5 μg purified THc via intramuscular (i.m.) routes. THc was