High-level Expression of the Hc Domain of *Clostridium botulinum* Neurotoxin Serotype A in *Escherichia coli* and Its Immunogenicity as an Antigen

A 型肉毒毒素 Hc 结构域在大肠杆菌中的高水平表达及免疫原性

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摘 要 对 A 型肉毒毒素受体结合区 *Hc* 基因的全序列进行优化和人工合成,获得了全长 1 287bp,编码 429aa 的 *Hc* 基因。以 pTIG-Trx 为原核表达载体,实现了 Hc 在大肠杆菌中的高效可溶性表达及纯化,该表达水平可占可溶性全菌总蛋白的 36% ~ 53%,经一步亲和层析纯化可获得电泳级纯度的目的蛋白,在常规培养条件下,产量达到 30mg/L 以上。然后,纯化的重组蛋白 Hc 免疫小鼠后能够诱导产生高滴度特异性的抗体,也能诱导产生特异性的细胞免疫应答反应。小鼠体内 A 型肉毒毒素中和试验结果表明免疫组小鼠血清中含高滴度的体内中和抗体。结果表明,利用本实验的原核表达系统不仅能够高水平可溶性地表达 A 型肉毒毒素受体结合区 Hc,而且重组 Hc 具有良好的免疫原性,可以用于制备治疗性抗毒素和作为亚单位候选疫苗用于预防 A 型肉毒毒素中毒。

关键词 肉毒毒素, 受体结合区 Hc, 可溶性表达, 免疫原性

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Abstract A completely synthetic gene encoding the Hc domain of *Clostridium botulinum* neurotoxin serotype A (*AHc*, 1287bp, 429aa, ~ 50kD) was constructed with oligonucleotides. After expressed in *Escherichia coli*, soluble product AHc was gained and verified by SDS-PAGE and Western blot analysis. The expressive level of recombinant AHc in *E. coli* was very high (36% ~ 53% of soluble total proteins) and the purified yield was more than 30mg/L by one-step purification. Then, the purified AHc was used to vaccinate Balb/c mice, which developed a strong and specific immune response as expected following administration of AHc protein via the subcutaneous route. Results from BoNT/A neutralization assay showed that the serum from mice vaccinated with AHc contained high titer protective antibody. These results showed that the soluble, stable and high-levelly expressive AHc not only could be produced by the prokaryotic expression system built in our lab, but also owned strong immunogenicity to prepare antitoxin for treatment and as sub-unit candidate vaccine for prophylaxis against botulinum toxin serotype A.

Key words botulinum neurotoxin, Hc domain, soluble expression, immunogenicity

As known the *Clostridial botulinum* neurotoxins (BoNT) produced by *Clostridium botulinum* are the most toxic substances, which can be divided into seven serotypes (A to G) with similar structure but distinct antigenicity. The botulinum neurotoxins exert their action by inhibiting the release of the neurotransmitter acetylcholine at the

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neuromuscular junctions and synapses causing potential death by flaccid paralysis[1]. Human are usually exposed to the botulinum neurotoxins through food poisoning and only there are rare incidents of wound botulism and colonizing infections in neonates known as infant botulism[2]. Human botulism is commonly associated with toxin serotypes A, B, E and F, in which serotype A is the most poisonous. Each of the toxins is a protein with an approximate molecular mass of 150kD, including a heavy chain (MW 100kD) and a light chain (MW 50kD) joined by a single disulphide bond. The toxin dimer may each be viewed as composed of three functional domains: The carboxyl-terminal 50kD of the heavy chain (Hc domain) of the toxin mediates binding to the target neurons, the amino-terminal 50kD of the heavy chain (H_N) is involved with internalisation of the toxins and the light chain identified as a zinc protease blocks neurotransmitter release[3,4].

Prevention of botulism can be efficiently achieved by vaccination, which generates neutralizing antibodies against botulinum neurotoxin. Currently, the most widely available vaccines for human are formalin-inactivated toxoid such as pentavalent toxoid (PBT). However, it is very expensive and time-consuming to produce and is hazardous during detoxification. To overcome some of the draw-backs of the toxoid vaccines, a recombinant sub-unit vaccine has currently been investigated[5]. While the complete holotoxin is required for biological toxicity, the Hc fragments (receptor binding domain, Hc domain) alone are nontoxic and antigenic and have demonstrated the ability to elicit a protective immune response in animals challenged with native botulinum toxin. The Hc fragments of serotypes A, C, D and F produced in *E. coli*[6-9] and the Hc fragments of serotypes A, B, C, E and F produced in *Pichia pastoris*[10-13] have been shown to elicit a protective immune response in mice. The part fragments of Hc (Hc-C) could also be expressed in *E. coli*[14,15] and possessed antigenicity and functionally active conformation, but these products were insoluble. More recently, the soluble Hc fragments of serotypes A and E successfully expressed in *E. coli*[16,17] also have been shown to elicit a protective immune response in mice. But, the yield of recombinant protein from *E. coli* in these studies was not high and required to be cultured for very long time. In addition, there are some defects on the first generation recombinant BoNT sub-unit vaccines from yeast expression system, such as very complex, expensive, and limited genetic manipulation.

Thus, it is very advantageous and valuable for us to easily and high-levelly produce the soluble Hc in *E. coli*, further to develop sub-unit vaccines against serotype A toxin. In current study we constructed a synthetic gene encoding the non-toxic 50kD carboxyl-terminal fragment (Hc domain) of botulinum toxin serotype A (*AHc*), expressed stable, soluble and high level AHc in *E. coli* and investigated the ability of AHc to elicit a strong and specific immune response. Now a more detailed study into the efficacy of the AHc fragment in protecting against toxin challenge is also in proceeding.

1 Materials and Methods

1.1 Bacterial strains and cloning vectors

DNA fragments which had been generated using the PCR were initially cloned directly into a T-tailed plasmid vector (pGEM-T, Promega), then transformed into *E. coli* strain DH5α. The synthetic AHc gene was cloned into expression vector pTIG-Trx (pTIG-Trx derived of pET-22b(+), and Trx gene was cloned into pET-22b (+) with *Nde* Ⅰ and *Eco*RⅠ) and the recombinant plasmid pTIG-Trx-Hc was expressed in *E. coli* strain BL21 (DE3).

1.2 Construction of AHc Gene

A synthetic gene (1287 nucleotide base pairs) encoding the Hc fragment of *Clostridium botulinum* neurotoxin serotype A (*AHc*, 868-1296aa, ~ 50kD) was firstly constructed as three blocks: A block was around 500bp, B 400bp, and C 400bp. A block was constructed from 18 overlapping oligonucleotides by overlapping PCR, both B and C from 14 overlapping oligonucleotides. The oligonucleotides were typically about 56 base pairs in length and contained complementary sequence at the 5′ and 3′ ends to allow annealing. The blocks were then made double stranded and further amplified using the PCR with LA Taq polymerase (TaKaRa). Blocks A ~ C were cloned into pGEM-T and sequenced. Blocks BC (800bp) was constructed from Blocks B and C, then Block ABC (full length *AHc* gene, 1287bp) was constructed from Blocks A and BC by fusion PCR and was cloned into pGEM-T. The correct clone containing AHc gene identified by sequencing was named pGEM-Hc.

1.3 Expression of AHc in *E. coli* and its purification

The oligonucleotide primers for PCR amplification of *AHc* gene using pGEM-Hc as template were designed as follows (the sequence underlined indicated enzyme recognition sites):

(1) F-HcE (*Eco*R Ⅰ): 5′-GCCGGAATTCTAAT

GGAATACATCAAGAACATCATC-3′,(2) R-HcX(*Xho* Ⅰ):5′-CTAGCTCGAGAGTGGACGTTCACCCCAAC-3′.

The PCR product was digested with *Eco*R Ⅰ and *Xho* Ⅰ and the excised fragment encoding AHc was cloned into suitably digested plasmid pTIG-Trx to create plasmid pTIG-Trx-Hc. The nucleotide of the cloned *AHc* gene was confirmed by sequencing to ensure authenticity. The recombinant plasmid was transformed into the *E. coli* strain BL21 (DE3), which was grown at 37℃ to an optical density at 600 nm of 0.5, induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) (final concentration, 0.2 mmol/L) at 30℃, and harvested after 3 h or 5 h by centrifugation. The cells were washed and resuspensed in PBS, then sonicated. The supernatant containing the soluble fraction was examined by SDS-PAGE and further purified. The expressive gene product containing a carboxyl-terminal hexahistidine tag was purified using Ni^{2+}-loaded HiTrap Chelating HP as recommended by the supplier (Amersham Biosciences). The soluble fraction and the purified AHc were monitored by SDS-PAGE and Western blot using hyperimmune horse toxin A antiserum (from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, china). Electrophoresis and semi-dry blot were carried out using the Bio-Rad system.

1.4 Immunization of mice

Six weeks old female Balb/c mice (SPF) were used in current study. Groups of eight mice were vaccinated with purified 1 ~ 10μg AHc protein. AHc were mixed 50∶50 with complete Freund's adjuvant (Sigma) for the first vaccination and incomplete Freund's adjuvant (Sigma) for the second and third vaccinations. Mice received 0.4mL of material via the subcutaneous route three vaccinations (day 0, 14 and 28). As a negative control, PBS instead of the antigen was mixed with the adjuvant. Mice were tail bled for sera prior to each injection and were eye bled for sera 14 days following their final vaccination.

1.5 Measurement of serum antibody titers

Serum from mice in each group was screened for antibodies specific to AHc by an ELISA. Test sera were initially diluted 1∶100 with BSA-PBST and serially diluted in duplicate. The total IgG titres and the individual isotype of IgG (IgG1 and IgG2a) were determined using HPR-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1∶2000. The adsorbance was read at 492 nm. The antibody titer was estimated as the maximum dilution of serum giving an absorbance reading greater than 0.5 units, after subtraction of the absorbance due to non-specific binding detected in control sera.

1.6 BoNT/A neutralization assay

Serum from mice 14 days following their final vaccination in each group was pooled for the toxin neutralization assay. The first test, the pooled serum samples were initially diluted 1∶10 and incubated for 0.5h at room temperature with 10 and 100 LD_{50} of botulinum toxin serotype A (from Lanzhou Institute of Biological Products, Lanzhou, china) and then injected into four mice via the intraperitoneal (i.p.) route. The mice were observed for 1 week, and death or not were recorded. The second test, the pooled serum sample from the group with 1μg dose AHc vaccination were initially diluted 1∶10 and then diluted tenfold to 1∶10 000 and incubated with 10 LD_{50} of botulinum toxin serotype A and injected i.p. into four mice as above.

1.7 Splenocyte proliferation

Spleens were removed from the immunized mice at 2 weeks after last immunization and homogenized in RPMI 1640. Splenocytes were plated in 96-well flat-bottom plates at 100μL per well. Subsequently 100μL per well of medium with or without recombinant AHc (10μg/mL) were added and mixed. Concanavalin A (5μg/mL, Sigma) was used as a positive control. Each splenocyte sample was plated in triplicate. The proliferative response was measured by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol. The stimulation index (SI) was calculated as the ratio of average *OD* value of wells containing antigen-stimulated cells to average *OD* of wells containing only cells with medium.

2 Results

2.1 Construction and cloning of synthetic AHc gene

The native *Clostridial botulinum* gene sequence has an A + T content of 76% and throughout the sequence there are a number of codons that are rarely employed in *E. coli* genes which might together result in very poor expression in *E. coli*[18]. To improve expression level of botulinum toxin gene, we designed a new *AHc* gene by reverse translating from the amino acid sequence according to the *Hc* of BoNT/A (A62), with the single most frequently used codon of *E. coli* for each amino acid to replace codons which were known to be found at low frequencies in *E. coli*. For construction synthetic *AHc* gene from synthetic oligonucleotides, it was first

divided into three blocks, and then full *AHc* gene was obtained by fusion PCR. The complete *AHc* gene (1287bp), designed to reduce the overall A + T content to 57.6%, would encode 429 amino acids (868-1296aa, about 50kD) at the C-terminus of the heavy chain of botulinum toxin A. For expression in *E. coli*, the synthetic *AHc* gene was cloned into plasmids pTIG-Trx to obtain plasmid pTIG-Trx-Hc.

2.2 Soluble expression of AHc in *E. coli* and its purification

AHc was expressed in *E. coli* (BL21) and was identified by its molecular weight and reaction with the antibodies in Western blots (Figs. 1 and 2). The results showed that the expression product about 50kD was soluble and the expression level was very high (more than 36% of total soluble protein, Fig. 1). Small scale purification of AHc was carried out by Ni-NTA affinity chromatography for the soluble protein. Expressed proteins, eluted from the Ni-NTA column with elution buffer (0.5mol/L imidazole), were analyzed by SDS-PAGE (Fig. 1) and Western blot and probed with anti-BoNT/A antisera (Fig. 2). Eluted fractions containing expressed proteins were pooled and dialyzed overnight at 4℃, against phosphate-buffered saline (PBS). Protein concentrations were estimated by using BCA (MERCK) according to the manufacturer's protocol. The approximate yield was more than 30mg/L culture only by one-step purification. The product was judged to be >95% pure by SDS-PAGE. In addition, the recombinant AHc product was full-sized according to SDS-PAGE and Western blot, and stable when stored at 4℃ for 1 months, −20℃, and −80℃ for 6 months (results not shown).

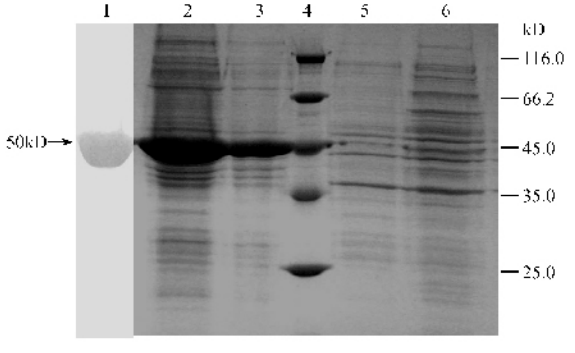

Fig. 1 SDS-PAGE of soluble AHc expressed in *E. coli*

After *E. coli* (BL21) cells were sonicated, the supernatant containing the soluble fractions were subjected to SDS-PAGE and Commassie blue stained. 1: purified AHc by Ni-NTA affinity chromatography; 2: the supernatant from cells transformed with pTIG-Trx-Hc vector and induced for 5h; 3: the supernatant from cells transformed with pTIG-Trx-Hc vector and induced for 3h; 4: low-molecular-weight protein markers; 5: the supernatant from cells transformed with pTIG-Trx vector; 6: the supernatant from cells transformed with pTIG-Trx-Hc vector, not induced. Molecular weights of the protein standards are indicated on the right (10^3). An arrow indicates the position of the AHc.

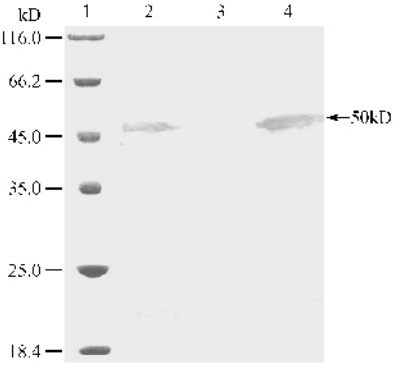

Fig. 2 Western blot analysis of soluble AHc expressed in *E. coli* and purified AHc by Ni-NTA affinity chromatography

1: low-molecular-weight protein markers; 2: the supernatant from cells transformed with pTIG-Trx-Hc vector; 3: the supernatant from cells transformed with pTIG-Trx vector; 4: purified AHc. An arrow indicates the position of the AHc.

2.3 Serum antibody titres

The serum total IgG titers of vaccinated and control mice against AHc were measured. The serum titers obtained in mice vaccinated with purified AHc of 1, 5, and 10μg are shown in Fig. 3. The group titer of mice vaccinated with a single dose of AHc of 1μg was just 3200, while the group titer of mice vaccinated with a single dose of AHc of 10μg was 12800 ($P = 0.010 < 0.05$). Following the second and third vaccination titers of three groups against the AHc were increased reaching a maximum of 204, 800, 409, 600, and 819, 200, respectively ($P = 0.0612 > 0.05$). The ELISA absorbance of the pooled serum from a negative control group was very low and the serum titers was <100 as the pooled

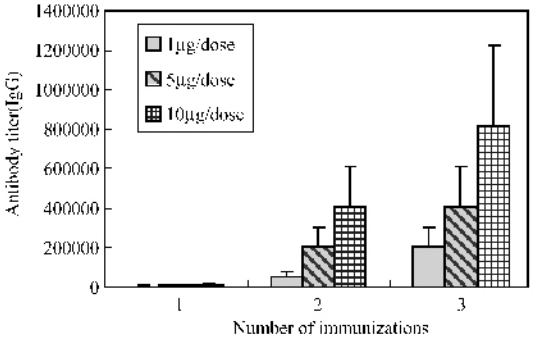

Fig. 3 Antibody titers of serum from Balb/c mice vaccinated (s.c.) with AHc of 1, 5, and 10μg

Total IgG titers against AHc were measured in serum samples taken prior to each injection and 14 days following their final vaccination. Serum samples from individual mice were assayed in duplicate, and the duplicate measurements were used to calculate the GMT ± SD for each group.

serum from mice before vaccination. In order to determine the nature of the immune response to AHc, isotype of the IgG response was carried out. The highest titer obtained was for IgG1 and IgG1/IgG2a was >30.

2.4 BoNT/A neutralization assay

To observe if serum from mice vaccinated with AHc contained protective antibody against BoNT/A, serum from mice vaccinated with three doses of AHc was used in a BoNT/A neutralization assay. The results of the first neutralization test showed that the pooled serum of the three groups could fully protected mice from challenge with 10 and 100 LD_{50} of botulinum neurotoxin serotype A. Thus, the titers of protective antibody of the pooled serum of the three groups were more than 2000 LD_{50}/mL. While the pooled serum from a negative control group could not protected mice from challenge with 10 LD_{50} of botulinum neurotoxin serotype A and mice dead within 12h. In order to further assay the titers of protective antibody from the pooled serum of the first groups vaccinated with purified AHc of 1μg, we carried out the second test. The results showed that the pooled serum of the first groups diluted 1∶1000 could fully protected mice from challenge with 10 LD_{50} of botulinum toxin serotype A and partly protected when diluted 1∶10000. Thus, the titers of protective antibody of the pooled serum sample from group with 1μg dose AHc vaccination were more than 20000 LD_{50}/mL. These results showed that the serum from mice vaccinated with AHc contained high titer protective antibody.

2.5 Proliferation of splenocytes

The presence of immune cells that can be directly stimulated by AHc was confirmed by measuring splenocytes proliferation *in vitro* in the presence of the antigen. Therefore, we also tempted to detect cell-mediated immunity from mice immunized with AHc in our study. Splenocytes from AHc-vaccinated and control group mice were analyzed at 2 weeks after final immunization. As shown in Fig. 4, the AHc-specific proliferative responses were significantly higher in mice immunized with different doses of AHc ($P = 0.4132 > 0.05$) than control group immunized with PBS ($P = 0.000348 < 0.01$). The results shown recombinant protein AHc vaccine induced significantly specific cell-mediated immune responses.

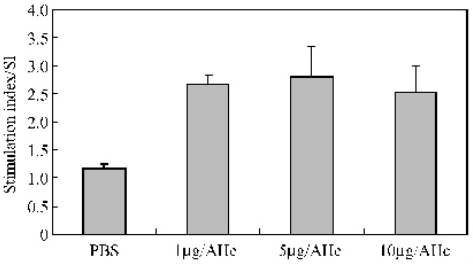

Fig.4 Proliferative responses of splenocytes from AHc-vaccinated mice after *in vitro* stimulation with recombinant AHc

Each group of mice ($n = 8$) was immunized with 1, 5, 10μg doses of AHc. At 2 weeks after final immunization, mice were sacrificed and splenocytes were stimulated with AHc proteins (10μg/mL). After 72h of stimulation, MTS were added and the adsorbance measured at 492nm/630nm were determined after a further 4h inoculation. Samples were assayed in triplicate. The ConA control samples showed a stimulation index of 4 ~ 6. Data represent the mean ± standard error.

3 Discussion

As an appropriate vaccine candidate, the carboxyl-terminal fragment of botulinum neurotoxin heavy chain (Hc) is responsible for toxin binding to the nerve cell and the recombinant immunogenic Hc has been proved to be an efficient antigen to generate neutralizing antibodies for immunotherapy. Based on the previous work we have proved that a synthetic gene encoding the carboxyl-terminal fragment from the heavy chain (Hc domain) of the botulinum neurotoxin serotype A could be high-levelly expressed in *E. coli* and maybe further exploited as sub-unit vaccines against the botulinum neurotoxin A.

As expressed in *E. coli*, our synthetic gene produced a protein product in a soluble form with the expected molecular mass (~ 50kD) on SDS-PAGE. The AHc product could react with antisera to BoNT/A in Western blot and ELISA. After mice were vaccinated with purified AHc, antibody titers and BoNT/A neutralization antibody of the pooled serum were detected by ELISA and BoNT/A neutralization assay, respectively. It was very encouraging that serum from mice vaccinated with AHc contained high titer protective antibody. In addition, Splenocytes proliferation was provoked by *in vitro* stimulation with AHc which indicated recombinant protein AHc vaccine induced significantly specific cell-mediated immune responses. In general, antibody plays a key role on protection against toxin and protection against toxin is largely mediated by antibody. Serum antibody titers or serum neutralization titers are commonly used to assess an animal's ability to survive challenge. Our results showed that serum from mice vaccinated with AHc contained high titer protective antibody and the higher titers induced was IgG1 isotype. The higher titers of IgG1 induced is consistent with a Th2 response which involves B lymphocyte activation and antibody

production.

Compared to other work on the Hc fragments of serotypes A, C, D, E and F produced in *E. coli*[6-9,14-17], the yield of soluble recombinant protein in current study was much higher (36% ~ 53% in *E. coli* soluble total proteins) and the purified yield was more than 30mg/L by one-step purification. In addition, expression of AHc in *E. coli* was cultured for only 3h at 30℃. So it is more advantageous and practical to produce and purify the recombinant protein AHc as a sub-unit vaccine at a large scale using our prokaryotic expression system. As suggested by our study, the soluble and high level expression of AHc was largely owe to the designed synthetic *AHc* gene and the pTIG-Trx expression vector used, because of the same synthetic *AHc* gene failing to express in other expresion vectors. The pTIG-Trx vector could express Trx together with AHc, so Trx could help AHc properly fold into correct conformation and express AHc in a soluble form. In addition, the length of *AHc* gene could affect properly fold into correct conformation that would not disrupt the neutralizing epitopes[5]. It was very encouraging that some problems existing in the expression of fragments derived of botulinum toxins such as AHc in *E. coli* were resolved in current study. The Hc domains of the other botulinum neurotoxin such as B, E and F have also been successfully expressed by this expression system in our lab now.

These results have shown the AHc product owned the real antigenicity and immunogenicity and serum from mice vaccinated with AHc could protect mice against i.p. toxin challenge. It is very possible for us to produce a sub-unit vaccine based on the Hc binding domain of botulinum neurotoxin serotype A or to prepare antitoxin for treatment.

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