INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-3, Issue-4, Oct-Dec-2012 Coden : IJABPT Copyrights@2012

ISSN : 0976-4550

Received: 13th Sept-2011 *Revised: 16th Sept-2012* *Accepted: 27th Sept-2012*

Research article

AN A-B SUBUNIT CHIMERA OF THE *E.COLI* VEROTOXIN 1 PROVIDES A RECEPTOR PROBE AND POTENTIAL VACCINE APPROACH.

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ABSTRACT: We have generated a chimera between the enzymatically active A subunit of the *E coli* derived AB5 verotoxin and a single receptor-binding B subunit. The construct was made by in frame fusion of the 3' terminus of the A subunit gene with the 5' end of the B subunit gene via the deletion of the intervening bases of the verotoxin operon such that the C terminus of the A subunit is continuous with the N terminus of a single B subunit. The gene product is a single fusion protein of 38kDa molecular weight, reactive with polyclonal and monoclonal antibodies against either the A or B subunits of the wild type toxin. The chimera showed a 104-105 fold reduction in cell vero cell cytotoxicity but no toxicity for the globotriaosyl ceramide (Gb3) deficient VRP subclone. No Gb3 binding by TLC overlay was detected. Polyclonal rabbit anti-VT1A-B chimera serum neutralizes VT1 cytotoxicity in vitro but reacts only with the A subunit of the wildtype holotoxin by western blot. This A-B chimera illustrates the importance of the pentameric B subunit in receptor binding and potentially identifies a novel attenuation vaccination strategy.

Key words: monomeric B subunit, overlap extension PCR, globotriaosyl ceramide binding, native holotoxin immunogen

INTRODUCTION

Verotoxins (VTs) are a family of protein toxins elaborated by verotoxin-producing *Escherichia coli* (VTEC). Originally described in 1977, they were appropriately named for their distinctive cytotoxic effect on African Green Monkey (Vero) cells (Konowalchuk, Speirs et al. 1977) and are also called Shiga toxins (Stx) since VT1 is virtually identical to the Shiga toxin from *Shigella dysenteriae* (O'Brien and LaVeck 1982). VT targets endothelial cells and are the primary cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS) which may follow gastrointestinal infection with VT producing *E.coli*(VTEC), particularly in young children and the elderly(Karmali, Petric et al. 1985; Karmali 2004). The more severe pathology (HUS) is more frequently associated with VT2 (Ostroff, Tarr et al. 1989; Boerlin, McEwen et al. 1999; Orth, Grif et al. 2007).

Structurally, verotoxins, like several other bacterial toxins, such as cholera and pertussis, have an AB5 subunit format (Middlebrook and Dorland 1984). VTs are composed of a single ~32kDa catalytic A subunit that is non-covalently inserted into the central pore created by the pentameric arrangement of five identical 7.7kDa B subunits (Fraser, Chernaia et al. 1994). The B-subunit mediates globotriaosylceramide (Gb3) glycosphingolipid (GSL) receptor recognition (Lingwood 1993). Each B-subunit monomer consists of two three-stranded anti-parallel s-sheets at the outer surface and an α helix which lines the central pore (Fraser, Chernaia et al. 1994).

Despite >20 years of study, VTEC infections remain a health threat worldwide, with no specific clinical interventive strategy as yet. A variety of carbohydrate-based(Dohi, Nishida et al. 1999; Kitov, Sadowska et al. 2000; Mulvey, Marcato et al. 2003; Pinyon, Paton et al. 2004; Nishikawa, Matsuoka et al. 2005; Watanabe, Igai et al. 2006; Neri, Nagano et al. 2007; Kitov, Mulvey et al. 2008) and non-carbohydrate based receptor analogues (Miura, Sakaki et al. 2006; Yamada, Miura et al. 2006; Stone, Hirama et al. 2007) have been developed which protect cells in vitro and mice in vivo against VT pathology but these have yet to prove clinically effective.

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B subunit (Marcato, Mulvey et al. 2001) or inactivated holotoxins (Bast, Brunton et al. 1997; Johansen, Andresen et al. 1997; Suzaki, Ami et al. 2002; Wen, Teel et al. 2006) have been used to generate neutralizing antibodies and proposed as a vaccine strategy. Expression of such toxins in plants may provide an innocuous delivery route (Wen, Teel et al. 2006). Human or humanized mouse neutralizing Mab provide a potential passive immunization protocol (Tzipori, Sheoran et al. 2004). Hybrid toxins for the simultaneous immunoprotection against VT1 and VT2 have been constructed (Smith, Teel et al. 2006). A fusion of the VT1 and VT2 B subunits has raised neutralizing antibodies against both toxins (Gao, Cai et al. 2009), an approach extended to a fusion protein comprising the inactivated VT2A subunit fused to the VT1B subunit (Cai, Gao et al. 2011). A (Cheng, Feng et al. 2009) or B(Oloomi, Bouzari et al. 2009) subunit fusion to other carrier proteins also generates an effective immunogen .

In our studies using a receptor analogue, we designed which was effective against VT1 and VT2 in vitro(Mylvaganam and Lingwood 1999; Tam, Mahfoud et al. 2008), VT2 pathology in vivo was increased(Rutjes, Binnington et al. 2002) suggesting that receptor analogues might function as pseudoreceptors. In order to design the most effective Gb3 analogues, detailed knowledge of the mechanism of Gb3 binding is needed. The exact nature of the B subunit Gb3 binding site however, remains unclear. Adjacent B subunit monomers interact to form a potential intersubunit cleft site (Stein, Boodhoo et al. 1992) which was identified in molecular modelling studies as the primary Gb3 binding site (Nyholm, Magnusson et al. 1996). In this site, Phe30 of one subunit and an aspartate loop of the adjacent subunit comprise the boundary of the binding cleft. A second, lower energy site on the other side of Phe 30 was also identified in this study (Nyholm, Magnusson et al. 1996). Subsequent cocrystalization studies with the lipid-free Gb3 sugar identified this second site, albeit with a different carbohydrate orientation, as the primary Gb3 binding site (Ling, Boodhoo et al. 1998). Subsequent VT1B site specific mutational studies indicated that although both sites were important for receptor binding on cells, site 2 was not the primary binding site which interacted with Gb3 glycolipid (Soltyk, MacKenzie et al. 2002), and in the VT2 crystal structure (Fraser, Fujinaga et al. 2004) this site is unavailable. Our studies have indicated the central importance of the lipid moiety of Gb3 in VT recognition (Boyd, Zhiuyan et al. 1994; Kiarash, Boyd et al. 1994; Binnington, Lingwood et al. 2002; Mahfoud, Manis et al. 2009) and a 5-6 log loss in VT1 binding affinity is seen for the lipid-free Gb3 sugar compared to the intact GSL(St. Hilaire, Boyd et al. 1994).

Several protein toxins which have a similar mechanism of toxic action as VT, eg ricin, A-Brin, comprise a format whereby the toxin is a single polypeptide chain and the A subunit is contiguous with a single receptor binding B subunit (Sandvig and van Deurs 1996). The polypeptide is proteolytically processed within the cell to release the A subunit from the B subunit, for membrane translocation and inhibition of protein synthesis. VT1 A subunit contains a C-terminal furin cleavage site which releases the activated A1 fragment for inhibition of protein synthesis and thus should remain active in a linear A-B fusion protein.

Since the amino acids which comprise site 2 of VT1 B are contained within a single B subunit, whereas site 1 requires the cooperation of adjacent subunits for Gb3 binding, it was considered that a fusion protein between the A and B subunits, preventing the pentameric B subunit association, would, if site 2 was utilized for Gb3 binding, retain Gb3 binding and cell cytotoxicity, while if site 1 was primarily utilized, Gb3 binding and cytotoxicity of the chimeric toxin would be severely compromised. Such a construct might then prove a useful attenuated immunogen to elicit protective antibodies against the native holotoxin structure.

MATERIALS AND METHODS

Construction of fusion protein gene splicing and overlap extension PCR

The VT1 A and B genes are found in adjacent operons separated by 9 bases. The A subunit has a 33 amino acid leader sequence and the B subunit contains its own promotor sequence within the signal sequence between these genes (Calderwood, Auclair et al. 1987).The first step is deletion of the 72 nucleotides in the signal peptide sequence between the A subunit and B subunit genes of VT1.

Primers The sequences of the four oligonucleotide primers used for this recombination are:

P1f(A):5'-GGGGGGATCCCACC**ATG**AAAATAATTATTTTTAGAGTGC-3' 39 nt

P2r(A):5'-TCCAGTTACACAATCAGGCGT***ACTGCTAATAGTTCTGCGCA***-3' 41 nt

P1f(B):5'-***TGCGCAGAACTATTAGCAGTA***CGCCTGATTGTGTAACTGG-3' 40 nt

P2r(B):5'-CTGAGCTATTCTGAATTC**TCA**ACGAAAAATAACTTCGCTG-3' 40 nt

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The reverse A subunit primer and the forward B subunit primer have a 20 base complementary extension sequence at their 3' and 5' termini respectively corresponding to the end of the A subunit (bold underlined).
The VT1 A and B gene fragments were separately amplified by specific designed primers from plasmid pJLB28, primers P1f(A), P2r(A) and P1f(B), P2r(B) for A and B subunits respectively.
The first round PCR products were purified by gel extraction (QIAGEN) and used as template to make whole fragment without signal sequence of B subunit in the second round PCR.
Overlap extension Primer P2r (A) and P1f(B) are splicing and overlap extension (SOE) primers, and they are used to modify the ends of the two PCR products so that they have the same sequence. When these PCR products are mixed, denatured and reanealed under PCR conditions. The top strand of the A subunit and the bottom strand of the B subunit sequences overlap and act as primers on one another. The recombinant product is formed when this overlap is extended by polymerase.

First round PCR

In a sterile 0.5 mL microfuge tube final concentration of the reaction buffer was 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH 8.3, 0.001% w/v gelatin, glycerol 5%, HifiTaq polymerase(Invitrogen) 0.4 unit per 10µl reaction, final concentration of each dNTP 200µM, 20µM of each primers P1f(A), P2r(A) and P1f(B), P2r(B), 20 ng pJLB28 plasmid DNA as template in both PCR reaction for A and B subunit amplification. Cycling parameters for A subunit: initial denaturation 94^0C 5 min, subsequent steps 94^0C 30 s, annealing at 55^0C 30 s, extension 72^0C 1 min, 25 cycles total, final additional extension 72oC 7 min, hold at 4^0C .

Cycling parameters for B subunit

initial denaturation 95^0C 5 min, subsequent steps 94^0C 30s, annealing at 50^0C 30s, extension 72^0C 1 min, 25 cycles total, final additional extension 72^0C 7 min, hold at 4^0C (Figure 4).

Second round PCR

In this step in a sterile 0.5 mL microfuge tube final concentration of the reaction buffer was 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH 8.3, 0.001% w/v gelatin, glycerol 5%, Taq polymerase (Genovision Inc.) 0.4 unit per 10µl reaction, final concentration of each dNTP 200µM, 20µM of each primers P1f (A), P2r(B) ,20 ng of purified A and B fragments as template in PCR reaction. Cycling parameters for second PCR: initial denaturation 94oC 2 min, subsequent steps 94^0C 30 s, annealing at 56^0C 1 min, extension 72oC 1 min, 30 cycles total, final additional extension 72^0C 7 min, hold at 4^oC.

Subcloning of recombinant fragment from second round PCR, transformation and Sequencing

In this step we used pDrive Cloning Vector (QIAGEN), supplied in a linear form with a U overhang at each end, which hybridizes with high specificity to the single A overhang at each end of PCR products generated using Taq and other non-proofreading DNA polymerases. Ligation protocol: pDrive Cloning Vector(50 ng/µl) 1 µl, PCR product (112 ng/µL) 4 µl, Ligation Master Mix, 2X (contains all reagents and cofactors required for ligation) 5µl, total volume was 10 µl, briefly mixed the ligation-reaction Mixture then incubate for one hour at 4 oC and transformed to the *E.coli* competent cells and plated on LB agar plate containing ampicillin (100 µg/mL) as selection marker For blue/white screening 40 µl of X-gal (40 mg/mL) was spread on the surface of the LB agar plate and incubated at 37 oC overnight and second incubation at 4^0C for few hours to enhance blue color development and thereby facilitates differentiation between blue and white colonies. The plasmids contain insert were verified by restriction enzyme digestion and sequencing.

Construction of expression plasmids

The pDrive plasmid containing the gene fusion insert was cut by EcoRI and inserted into pET28c expression vector (Novagen).The DH5a E.coli strain was used for initial cloning of target DNA into pET28c vector and for maintaining plasmids because they are recA$^-$ endA$^-$ and have high transformation efficiency and good plasmid yields. The new constructed plasmid was sequenced and named pETnkb-A-B.

Transformation into the expression host

Host BL21-DE3 *E.coli* strain was transformed with pETnkb-A-B. Strain BL21 (DE3) which is a lysogen of a bacteriophage DE3, a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the lacI gene, the lacUV5 promoter and the gene for T7 RNA polymerase, lacUV5 promoter is inducible with isopropylthiogalactoside (IPTG).The plasmids contain insert were verified by restriction enzyme digestion and sequencing.

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An overnight culture from a single freshly streaked plate was incubated with shaking at 37oC until OD600 reached 0.6. The cells were induced with 0.4 mM IPTG and the incubation continued for 3 hours, when the cells were pelleted at 5000 xg for 5 min. The pellet was resuspended in 0.25 culture volume of cold 20 mM Tris-HCL pH 8.0, and A 250 mL LB supplemented with 30 µg/mL kanamycin was inoculated with 1 mL of centrifuged as A-Bove.
A protease inhibitor cocktail tA-Blet (Roche) and 0.2 mg/mL colymycin wre dissolved in 50mL PBS. Five mL of cold protease inhibitor solution plus colymycin was added to each gram of cell pellet, vortexed vigorously and incubated at 37^0C for 1 hour. Cells were recovered by centrifugation and supernatant retained as periplasmic fluid. The pellet was resuspended in 5 mL fresh extraction buffer without colymycin and broken by sonication for 20 s, three times in an MSE Ultrasonic Disintegrator (John Scientific, Toronto, ON.). Cells were centrifuged and supernatant retained as soluble fraction. The pellet was resuspended in 1/50 culture volume SDS–sample buffer and boiled for 3 min with vortexing and collected as soluble plus insoluble fraction. Periplasmic fluid and soluble fraction extracts were used for SDS/PAGE Western blot analysis.

Purification of A-B fusion protein by µMACS His Tagged Protein Isolation

(Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The MicroBeads bind specifically to the His epitope of the target protein. The magnetically-lA-Beled proteins are retained on a µ Column placed in the magnetic field of a µMACS Separator according to manufacturer's guidelines. To magnetically separate the epitope-tagged target protein, 50 µL anti-His MicroBeads were added to the bacterial lysate, mixed well and incubated for 30 minutes on ice. After the lA-Belling incubation was finished, the cell lysate was applied onto the column. The column was rinsed with 4x200 µL of wash buffer 1 (150mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris HCl pH 8.0) and then rinsed with 1x100 µL wash buffer 2 (20mM Tris HCl pH 7.5). The native protein was eluted by pH shift. 20µL of 0.1M triethylamine, pH 11.8, 0.1% Triton X-100 was applied to the column and incubated for 5 minutes at room temperature. Then 50µL of 0.1M triethylamine, pH 11.8, 0.1% Triton X-100 was applied to the column and the eluate collected in a tube containing 3µL of 1M Mes, pH3 for neutralization. The last step was repeated twice and the eluates were collected in separate fractions and analysed by SDS-PAGE and Western Blot.

Immunoblot Analysis

Total cell extracts were prepared by boiling cell pellets for 10 min in reducing SDS–sample buffer. Equal protein aliquots were separated on 12% SDS–tricine gels and transferred to nitrocellulose membranes, blocked with 5% (w/v) nonfat milk in 50mMTrisbuffered saline (TBS) for 1h, and then washed in TBS. Membranes were incubated with rabbit polyclonal anti-VT1A (raised against HPLC separated A subunit from dissociated VT1 (Head, Karmali et al. 1991)) which reacts with all VT A subunits, or rabbit polyclonal anti-VT1 B antiserum (Boyd, Richardson et al. 1991) in TBS for 1h, washed extensively, then incubated with goat anti-rabbit horseradish peroxidase conjugated antibody (Bio-Rad) for 0.5h. Membranes were washed and developed with 4-chloro-1-naphthol in 50 mM TBS plus 3% (v/v) hydrogen peroxide.

Crosslinking

9 µg of A-B fusion protein or 10µg VT1 were cross-linked with dimethylsuberimidate (final concentration, 5 mg/ml) in 0.5 triethanolamine, pH 8.5 (Head, Karmali et al. 1991). The dimethylsuberimidate was allowed to react with the protein for 19 hours, without stirring, at room temperature. Sodium dodecyl sulfate (SDS) and β- mercaptoethanol were added, both to final concentration of 1%, and the solutions were further incubated for 2 hours at 37^0C. 150 µl of sample were than mixed with 50 µl of 0.01% bromophenol blue in 50 % glycerol. The cross-linked products were then separated by SDSpolyacrylamide gel electrophoresis using the Tricine method (Schagger and von Jagow 1987) for resolution of proteins less than 100 kDa. Protein bands were visualized by silver staining.

Vero cell cytotoxicity assay

Vero cells were adjusted to 75000 cells/mL media and 200mL were pipetted into each well of a 96-well plate. Cells were then incubated overnight at 37oC, 5%CO2 to allow them to adhere to the plate. VT1 or purified VT1A-B were titrated serially in media using 10-fold dilutions and 50 ml of each dilution was added to the monolayer of cells. After 3 days of incubation at 37oC and 5%CO_2, media was removed from the wells. Attached cells were fixed onto wells using a 2% formalin/PBS solution, incubated for 1 minute and then the solution was discarded. Fixed cells were then stained with crystal violet (0.125% in 5% ethanol, 2% formalin in PBS) for 30 minutes. Wells were washed, air dried, and 10% acetic acid (100mL/well) was added to disperse the dye. Absorbance at 560nm was measured as an index of cell viability.

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Neutralization of VTl cytotoxicity was monitored by preincubation of VT1 (2x LD50) with increasing dilutions of polyclonal anti VT1A-B for 1hr and assay of residual cell killing.

TLC overlay Gb3 binding assay.

Purified GSLs (SGC 1.5mg, GM1 1mg, LC 1mg, Gb3 1mg, Gb4 1mg) (dissolved in chloroform: methanol, 2:1) were separated on silica-coated plates (Macherey-Nagel) (C:M:W 65:25:4 v/v). Plates were blocked overnight in 1% (w/v) gelatin at 37°C and then washed in 50 mM TBS. Plates were incubated 1.5 h at room temperature with 3.7mg VT1 or 64.4mg of bacterial cell extract in 5mLTBS. Plates were washed and then incubated with a 1:1000 dilution of polyclonal anti-VTA for 1.5h, washed again, and incubated for 1.5h with a 1:2000 dilution of goat anti-rabbit horseradish peroxidase-conjugated antibody (Bio-Rad). Plates were developed in 4-chloro-1-naphthol developer until bands reach a stA-Ble intensity.

Rabbit immunization

New Zealand White rabbits were injected intramuscularly with 0.15µg purified VT1A-B in complete Freunds adjuvant and boosted with a second injection in incomplete Freunds after 1month. Serum was collected 10days later.

RESULTS

Generation of VT1A-B subunit chimera A schematic for the VT1A-B subunit fusion protein to be generated is shown in figure 1. The A and B subunit genes separately amplified using the extension primers are shown in Figure 2A,B. The fusion product was generated when the overlapping strands of the A and B product were used as primers (Figure 2C).

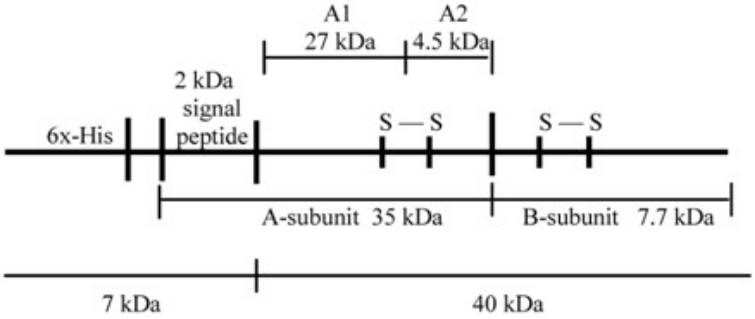

Fig 1 Schematic of VT1A-B subunit fusion protein. The 6xHis tagged fusion protein to be generated and the molecular weight of its component parts is shown.

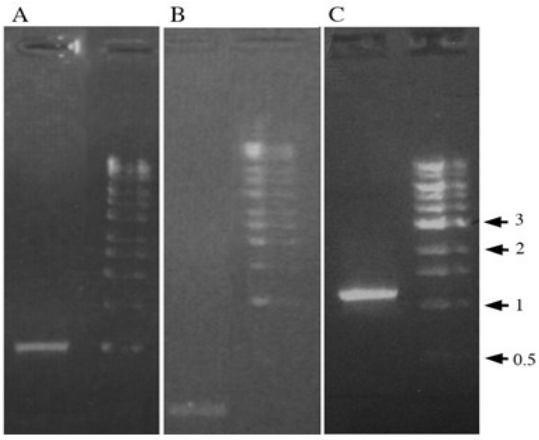

Fig 2 Fusion of VT1 A and B genes by overlap extension. Panel A: PCR amplification of VT1 A gene using P1f(A) and P2r(A) primers; Panel B: amplification of VT1 B gene using P1f(B) and P2r(B) primers; Panel C: second round PCR using P1f(A) and P2r(B) primers to amplify 1234bp A-B fusion product. 1kbDNA ladder in right lane of each panel and kb indicated to the right.

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The A-B fusion protein was expressed in *E.coli* and detected as an anti VT1 A reactive species (Figure 3) of apparent molecular weight 38 kDa, which was purified via anti-His affinity separation (Figure 4). VT1A-B was detected by western blot by both anti VT1A and anti VT1B antibodies (Figure 5).

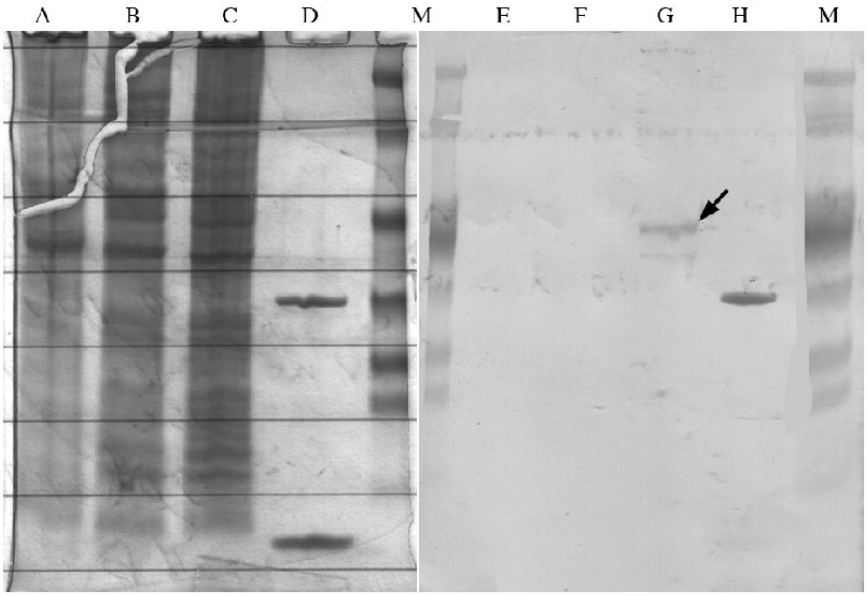

Fig 3 *E. coli* expression of A-B fusion protein. Coomassie blue stained SDS-PAGE (lanes A-D) and Western blot analysis using anti VT1 A (lane E-H). Lanes A and E *E.coli* BL21 without expression vector, Lanes B and F BL21(DE3) with pET-nkbA-B (vector plus insert), C before, and G three hours after induction with 1 mM IPTG. Fusion product is arrowed. Lanes D and H are Verotoxin1. M - protein size markers(11, 17, 26, 34, 43, 55, 72, 95, 130, 170 KDa).

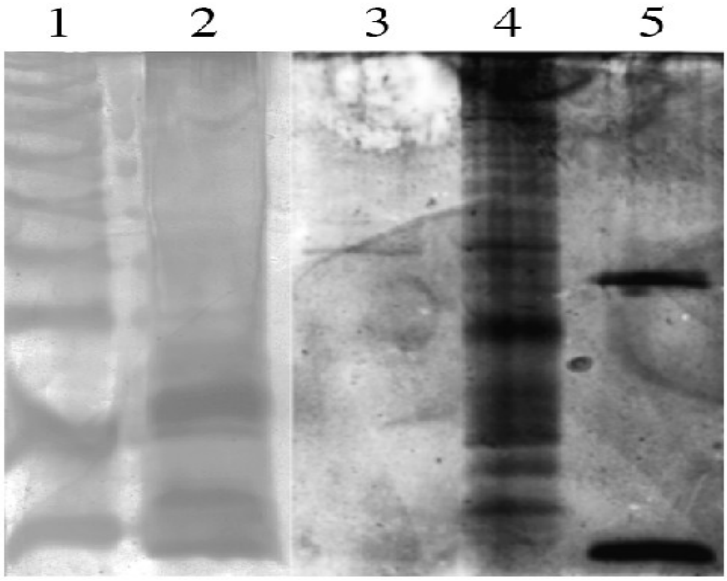

Fig 4 Purification of fusion protein. The isolated A-B fusion protein was separated by 15% SDS-PAGE and silver stained. Lane 1, molecular weight protein standard markers, lane 2, bacterial lysate; lane 3, 0.6 µg pure A-B fusion protein; lane 4, Impure A-B protein; lane 5, 2 µg VT1 toxin.

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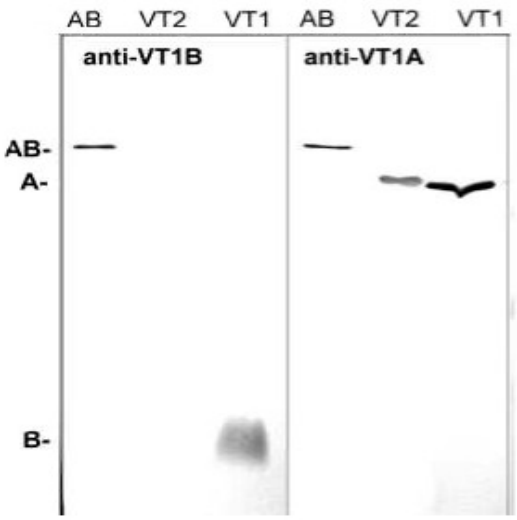

Fig 5 Immunoreactivity of VT1A-B fusion protein. The reactivity of VT1 A-B was compared by western blot to that of VT1and VT2 using polyclonal rabbit anti VT1 B subunit, anti VT1 A subunit.. Anti VT1 A subunit showed some cross reactivity with VT2 A subunit. The chimeric VT1A-B reacted with both A and B subunit specific antibodies.

Unlike the VT1 holotoxin, chemical crosslinking with dimethylsuberimidate had no effect on the molecular weight of this chimera (figure 6), consistent with VT1A-B expression as a monomer.

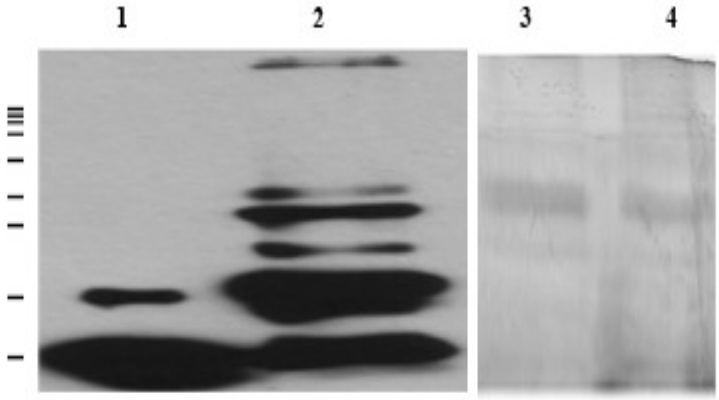

Fig 6 Crosslinking of A-B fusion protein.

Dimethylsuberimidate crosslinking of VT1 and the VT1A-B chimera was compared. Untreated VT1 (lane 1) or suberimidate treated VT1 (lane 2) or A-B chimera (lane 3) or suberimidate treated A-B chimera (lane 4) were separated by SDS-PAGE and detected by anti VT1B western blot. Molecular weight markers are indicated left. Unlike VT1, the molecular weight of the A-B chimera was unchanged after the crosslinking procedure.

Cell Cytotoxicity The purified A-B fusion protein was at least ~50,000 times less cytotoxic for Vero cells than VT1 holotoxin (Figure 7). Gb3 deficient VRP vero cell mutants (Pudymaitis, Armstrong et al. 1991) were resistant to VT1A-B (Figure 4b). Rabbit anti-B subunit antiserum showed complete neutralization of VT1A-B Vero cells cytotoxicity.

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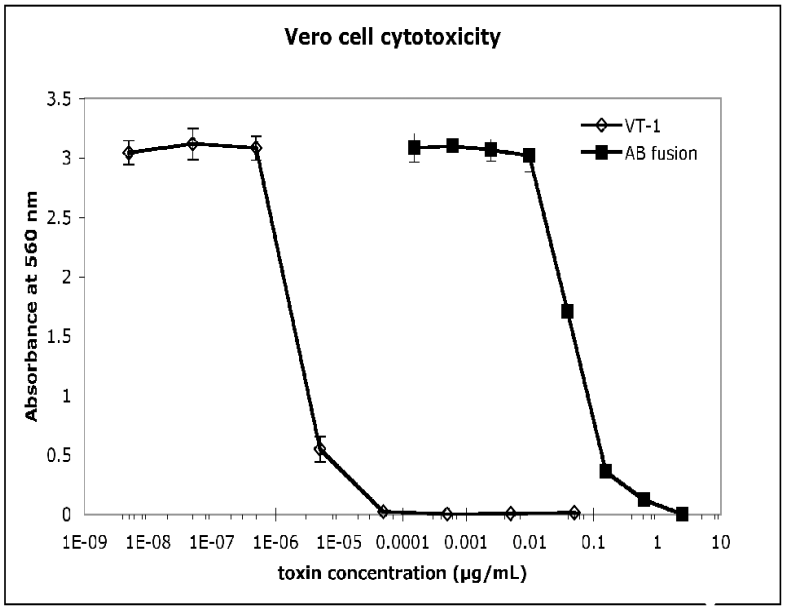

Fig 7 Comparison of VT1 and VT1A-B fusion cytotoxicity.

VT1 and the A-B chimera were titred out on to vero cells and residual cells stained with crystal violet after 72hrs culture. Cytotoxicity of VT1A-B was reduced by 5 orders of magnitude compared to VT1.

Gb3 glycolipid binding VT1A-B Gb3 binding could not be detected by TLC overlay (Figure 8) at concentrations in excess of 100 fold that necessary to detect VT1/Gb3 binding.

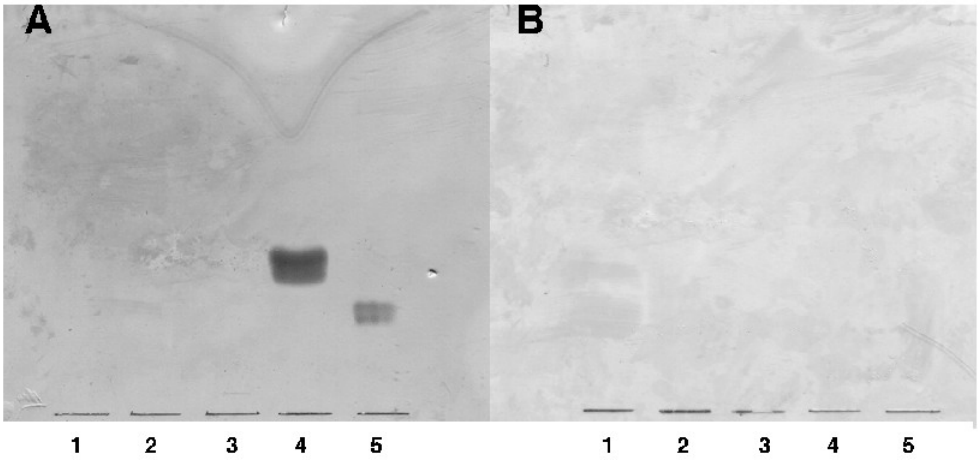

Fig 8 GSL binding of A-B fusion protein by TLC overlay VT1(0.1µg/mL) and VT1AB(13µg/mL) binding to the Gb3 receptor GSL was compared by TLC overlay. Panel A VT1, Panel B VT1 A-B. GSLs: lane 1 SGC, lane 2 GM1, lane 3 Gb3, lane 4 Gb4. VT1 binding to Gb3 (and a little to Gb4) was readily detectA-Ble but no GSL binding was detected for VT1A-B.

VT1A-B immunogenicity Rabbit polyclonal antibodies raised against VT1A-B reacted with VT1 but not VT2. However, these antibodies detected the A but not the B VT1 subunit (Figure 9). These antibodies could neutralize VT1 cytotoxicity in vitro (figure 10).

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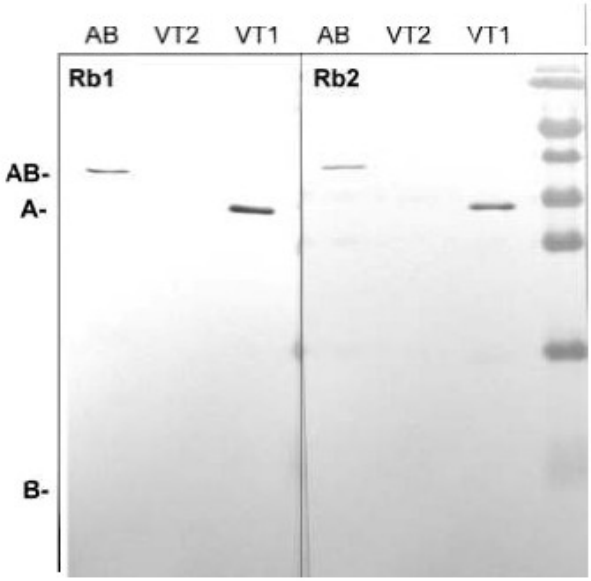

Fig 9 Immunogenicity of VT1A-B Sera from 2 rabbits immunized with VT1A-B(Rb1,Rb2) were tested by western blot for reactivity against VT1A-B, VT2 and VT1. Protein size markers(11, 17, 26, 34, 43, 55, 72, 95, 130, 170 KDa) are shown right. Anti VT1 A-B bound VT1A-B but did not react with VT2 A or B subunits and only recognized the A subunit of VT1.

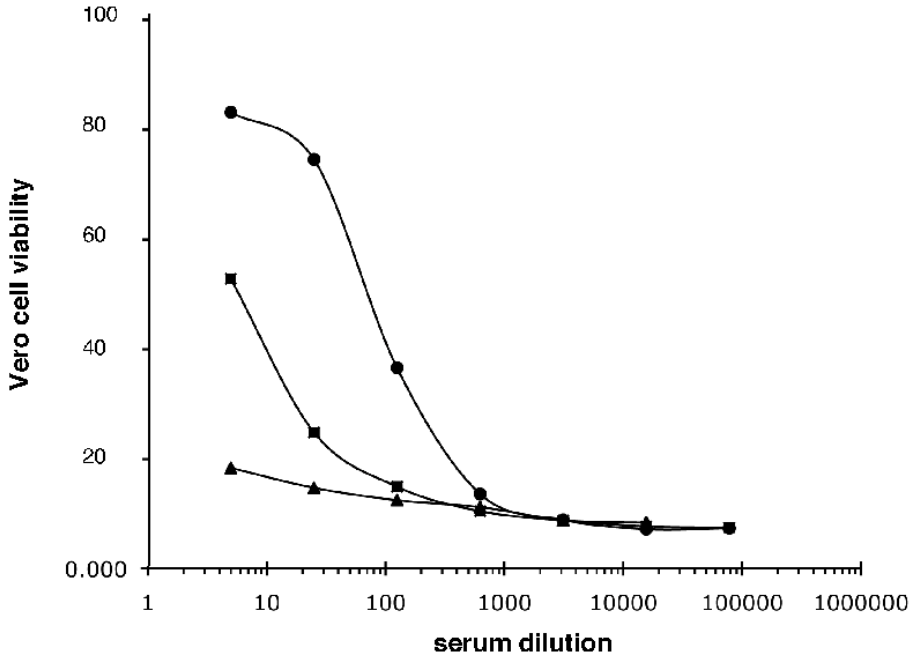

Fig 10 AntiVT1 A-B neutralization of VT1 cytotoxicity. VT1 was preincubated with increasing dilutions of rabbit polyclonal anti VT1A-B serum. Residual vero cell cytotoxicity was measured after 72hr.Preimmune serum ▲, rabbit 1 ●, rabbit 2 ■.

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DISCUSSION

These studies establish the feasibility of generating a linear fusion polypeptide containing both the A subunit and the B subunit of the A-B5 subunit verotoxin 1, to probe the mechanism of action of this bacterial virulence factor. The chimeric toxin has an apparent molecular weight (38kDa) consistent with a polypeptide containing the full length A and B subunits. This polypeptide is fully reactive (as compared to the native toxin) with polyclonal antibodies to both the A and B subunits as monitored by SDS/PAGE/western blot.

This immunoreactivity is detected against the denatured protein and does not define whether the native fusion polypeptide folds in a manner which preserves all the antibody binding epitopes of the separate native toxin subunits. Although the specific activity of the fusion protein is much reduced, VT1A-B retains partial cytotoxicity against vero cells, the standard cells index of VT cytotoxicity. This toxicity is fully neutralized by polyclonal and monoclonal anti VT1 antibodies, indicating that the fusion protein does indeed retain the native conformation of the separate A and B subunits. This residual cytotoxicity of the fusion protein was not seen for VRP mutant vero cells, which are deficient in Gb3 (Pudymaitis, Armstrong et al. 1991), indicating that a low level Gb3 recognition is retained in the recombinant chimera.

However, using TLC overlay, we were unA-Ble to detect Gb3 binding using the fusion protein. This is likely a reflection of the lower sensitivity of this solid phase binding assay. For VT1, 0.1µg/mL is sufficient to detect Gb3 binding, but for VT1A-B no binding was detected at 13µg/mL. Thus, the fusion protein shows a major reduction in Gb3 binding.

From the VT1B subunit crystal structure, the Gb3 binding site (site 1) was proposed to be within the intra-B subunit cleft(Stein, Boodhoo et al. 1992). This was supported by molecular modeling (Nyholm, Magnusson et al. 1996) which showed a potential additional, lower affinity binding site (site 2) within a trough on the membrane apposing surface of a single B subunit(Nyholm, Magnusson et al. 1996). Cocystalization with the Gb3 oligosaccharide identified site 2 as the primary carbohydrate binding site and site 2 was secondary, together with a 3rd site comprising trp 34(Ling, Boodhoo et al. 1998), implicated in multimeric receptor binding(Soltyk, MacKenzie et al. 2002). Although mutational analysis identified each of these three sites as important for cytoxicity(Bast, Banerjee et al. 1999), it remains unclear as to whether site 2 is the primary Gb3 glycolipid binding site(Soltyk, MacKenzie et al. 2002). Significant binding differences between Gb3 glycolipid and lipid-free Gb3 oligosaccharide have been reported (Boyd, Magnusson et al. 1994; St. Hilaire, Boyd et al. 1994; Soltyk, MacKenzie et al. 2002).

The major reduction in cell cytotoxicity and Gb3 binding seen for the VT1A-B chimera is primarily consistent with site 1 in the B subunit pentamer being the major site responsible for Gb3 glycolipid binding. Since this site is defined by amino acids within the n and n+1 adjacent B subunits (Nyholm, Magnusson et al. 1996), it will be lost in this construct.

In contrast, site 2 is contained, for the most part (10/11 H bonds(Ling, Boodhoo et al. 1998)) within one B subunit, as is site 3 (trp34) and should not therefore, be greatly affected by the monovalent nature of the B subunit within the chimeric toxin. Nevertheless, VT1A-B retained low level Gb3-dependant cytotoxicity, indicating that site 2 can bind cell membrane Gb3 (less effectively). Moreover, the effect of avidity loss due to monovalent, as opposed to pentavalent binding, has yet to be determined. Cross-linking studies established that VT1A-B does not oligomerize in any way. Thus the low residual cytotoxicity of VT1A-B is likely due to site 2 (+site 3?) binding. The reduced cytotoxicity of VT1A-B allowed rabbit immunization with the native polypeptide without adverse effect. A robust IgG immune response was observed and immune serum was effective to neutralize VT1 vero cell cytotoxicity. However, the anti VT1A-B antibodies generated were reactive only with the VT1 A subunit. Since VT1A-B cytotoxicity could be neutralized by anti VT1 B anti-serum, the lack of anti B subunit activity in the anti VT1A-B serum, suggests that the B subunit of the chimera might more optimally present the native A subunit immunogen, Alternatively the minimal residual Gb3 binding of the chimera may be sufficient to target dendritic cells, a process which has been previously used to advantage in B subunit conjugate immunogens (Haicheur, Benchetrit et al. 2003; Vingert, Adotevi et al. 2006).

ACKNOWLEDGEMENTS

This work was supported by and Iranian scholarship to N.K-B and CIHR grant number MT 13747.

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