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Human Monoclonal ScFv That Inhibits Cellular Entry and Metalloprotease Activity of Tetanus Neurotoxin

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SUMMARY Tetanus is a deadly disease of warm blooded animals and humans caused by an exotoxin called tetanospasmin or tetanus neurotoxin (TeNT) produced by anaerobic bacterium named *Clostridium tetani.* TeNT is an A-B toxin; each molecule consists of a heavy chain (HC) containing cellular receptor binding domain and a light chain (LC) with zinc metalloprotease activity. TeNT produced in the infected tissue by the bacteria grown under anaerobic condition binds to ganglioside receptors of peripheral nerve, and endocytosed. The A subunit exits from the endosome and undergoes a retrograde transport *via* the nerve axon to the spinal cord. This highly toxic enzyme specifically cleaves one of the nerve cell SNARE proteins, *i.e.*, synaptobrevin, resulting in inhibition of the release of neurotransmitters (glycine and GABA) from inhibitory interneuron causing spastic paralysis, the characteristic of tetanus. Current treatment mainstay of human tetanus is by passively administering anti-tetanus toxin produced from animals immunized with adjuvanted tetanus toxoid (TT). There are several obstacles in production and use of the animal derived therapeutic antibody especially the allergic reaction and serum sickness induced by the host immune response to the foreign protein. The animal antibody, mainly IgG, blocks nerve cell entry of the TeNT but does not neutralize the TeNT protease activity *per se* and cannot reverse the tetanus symptoms. In this study, fully human single chain antibody fragments (HuScFv) were produced from a human antibody phage display library. TT was used as antigen in a single round phage bio-panning to select phage clones that display TT bound-HuScFv from the library. HuScFv from 4 selected *huscfv*-phagemid transformed *E. coli* clones inhibited binding of the native TeNT to retinoic acid pulsed human neuroblastoma cells when used at the molecular TeNT:HuScFv ratio of 1:100. HuScFv from one of the 4 clones also inhibited the TeNT mediated cleavage of recombinant synaptobrevin. Further investigation is needed for identification of epitope specificity of these HuScFv and HuScFv effector mechanisms towards the TeNT. Cell penetrating version of the HuScFv that inhibited the TeNT zinc metalloprotease activity should be made. The HuScFv produced in this study either singly or in their suitable combination warrant developing further to a real use in humans as a surrogate of the animal antibody for treatment of tetanus.

Tetanus is a disease of humans and warm blooded animals caused by a bacterial protein toxin, named tetanospasmin or tetanus neurotoxin (TeNT) which is produced by *Clostridium tetani* grown under an anaerobic condition. *C. tetani* is ubiquitous and humans usually get the infection *via* punctured wounds contaminated with the bacterial spores. Anaerobic condition within the deep wound facilitates the spore germination proper, bacterial growth, and production and release of the TeNT. TeNT is an A-B type of toxin with a molecular mass of 150 kDa. The polypeptide is nicked by the bacterial pro-

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tease to yield a 50 kDa (A or light chain) subunit with zinc metalloprotease activity and 100 kDa (B or heavy chain) subunit which are still linked together by non-covalent interaction.[1,2] TeNT produced by the infecting *C. tetani* at the site of infection binds to receptor, *i.e.,* ganglioside on the peripheral nerve cell and endocytosed.[3] The A subunit exit from the endosome and undergoes a retrograde transport *via* the nerve axon to the spinal cord.[4] This highly toxic enzyme mediates inhibition of the release of neurotransmitters (glycine and GABA) from the inhibitory interneurones into the synaptic cleft resulting in an inability of the contracted muscles to relax and thus causing spastic paralysis, the characteristic of tetanic spasm (opisthotonusm, risus sardonicus, and locked jaw). Specific substrate of the A subunit zinc metalloprotease is one of the nerve cell SNARES proteins, *i.e.,* vesicle associated membrane protein-2 (VAMP-2) or synaptobrevin (Syb2).[5,6] Tetanus causes high mortality if not treated properly due mainly to the respiratory failure. [7,8]

Current management of a tetanus patient is supportive such as by providing an artificial respirator as well as passively giving anti-TeNT serum or polyclonal antibodies (PAb) derived from animal such as horse which had been immunized with adjuvanted tetanus toxoid. The PAb blocks cellular entry of the toxin but does not neutralize the endanger endopeptidase activity *per se*.[9] Complications, *e.g.,* IgE- and/or immune complex- mediated hypersensitivity (serum sickness) usually develop in the patients treated with the antibody of the heterologous species.[10,11] Other limitations of the animal derived immune serum include prolonged immunization procedure, risk of the recipient to certain zoonosis, and a batch-to-batch variation in the therapeutic efficacy of the antiserum. Fully human specific antibody to the TeNT which can be produced *in vitro* without the immunization process should be a better alternative of the animal derived immune serum.[12,13] Thus in this study, fully human antibody in the version of single chain variable fragments (ScFv) that bound specifically to the tetanus toxoid (TT) were produced *in vitro* from an established human antibody phage display library. The engineered human ScFv (HuScFv) were tested for their ability to inhibit cellular entry and zinc metalloprotease of the native TeNT. Details of the experimental design and results are reported herein.

MATERIALS AND METHODS

Human antibody phage display library

A naïve human antibody phage library used in this study was constructed in our laboratory from immunoglobulin genes of 60 naïve Thai blood donors. The details of the construction, the repertoire of the antibody diversity, and the other attributes of this phage library have been described elsewhere.[14,15] Briefly, genes encoding all families of human VH and VL immunoglobulin sequences were PCR amplifies using 42 pairs, *i.e.,* 14 forward x 3 reverse, of degenerate primers. The *VH* and *VL* amplicons were molecularly linked at random *via* a polynucleotide linker to generate a repertoire of *VH*-linker-*VL* or *scfv.* The human *scfv (huscfv)* sequences were cloned into pCANTAB5E phagemid vectors and the recombinant phagemids were introduced into competent suppressor TG1 *E. coli* cells. The phagemid transformed bacteria were grown and the mature phage particles displaying HuScFv on the surface with the respective *huscfv* in the phage genome were rescued by co-infecting the bacteria with M13KO7 helper phages. After co-infecting the TG1 bacteria with the helper phages, ~ 6 x 10^{12} pfu of complete phage particles were obtained. More than 80% of phages in the library contained *huscfv* sequences in their genome. Epitope diversity of the library was ~ 2.6 x 10^{8}. The rescued library had ~50 phage particles that displayed HuScFv to individual epitopes.

Tetanus neurotoxin (TeNT) and tetanus toxoid (TT)

TeNT used in this study was purchased from Sigma Chemical Company, USA. Tetanus toxoid (TT) was purified from the commercially available alum adsorbed tetanus toxoid (Bio Farma, Indonesia). In order to remove the adjuvant, the preparation was centrifuged at 12,000 x *g*, 4°C for 20 minutes. The supernatant was dialyzed against distilled water at 4°C to remove ions and salts. The TT was lyophilized and the protein content was determined.[16,17]

Preparation of fully human single chain antibody fragments (HuScFv) to tetanus toxoid

The TT was used as an antigen in the phage bio-panning for selecting phage clones displaying

HuScFv that bound to the TT from the phage library. The phage bio-panning of this study was performed essentially the same as previously described.[14,18] Briefly, one microgram of the purified TT in 100 µl of carbonate-bicarbonate buffer, pH 9.6, was added to each well of a polystyrene microplate (Costar, USA) and the plate was kept at 4°C overnight. After extensively washing all wells with phosphate buffered saline, pH 7.4 (PBS) containing 0.01% Tween-20 (PBST) to removed unfixed materials, the empty sites on the well surface were blocked by adding 200 µl of 3% bovine serum albumin (BSA) to each well and the plate was kept at 37°C for 1 hour. Excess BSA was washed off and 50 µl of the human antibody phage display library containing approximately 10^{11} pfu were added to each well. Binding of the phages to the immobilized TT was allowed to occur at 37°C for 2 hours. Unbound phages were removed by washing extensively with the PBST. A volume of a log phase grown non-suppressor HB2151 *E. coli* was added to each well containing the TT bound phages. Phage transduction was allowed to occur at 25°C for 20 minutes. The content of each well was then spread onto a surface of a 2x YT agar plate and the plate was incubated at 37°C overnight. *E. coli* colonies with recombinant *huscfv*-phagemids were screened by PCR using *R1* and *R2* primers.[14] The expected size of the *huscfv* was approximately 1,000 base pairs (bp). For producing soluble HuScFv, each clone of the *huscfv*-phagemid transformed *E. coli* was grown under IPTG induction condition.[14] The bacterial cells were collected by centrifugation and the soluble HuScFv were purified from the bacterial lysate by using DEAE Sepharose resin as previously described.[18]

Restriction fragment length polymorphism (RFLP) of *huscfv* sequences from transformed *E. coli* clones

RFLP patterns of *huscfv* sequences from the *huscfv*-phagemid transformed *E. coli* clones that could express soluble HuScFv were determined by digesting the PCR amplified *huscfv* sequences with the *Mva*I restriction endonuclaease.[15] The digested DNA was subjected to 12% polyacrylamide gel electrophoresis and ethidium bromide staining. The DNA banding patterns were recorded using a Gel Documentation System (Syngene, Cambridge, England).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (WB)

SDS-PAGE was performed for determining the purity of TT using 12% polyacrylamide gel and the protein bands were stained with Coomassie Brilliant Blue R-250. WB was used also for revealing the HuScFv in *E. coli* lysates, studying the zinc metalloprotease activity of the TeNT and determining the capacity of HuScFv in inhibiting the endopeptidase activity of the TeNT in a band shift assay. In the WB, the SDS-PAGE separated components were blotted onto a nitrocellulose membrane (NC); the NC was blocked with BSA solution and probed with primary antibody, *i.e.*, mouse monoclonal anti-E-tag for detection of E-tagged-HuScFv and rabbit anti-human VAMP1/2/3 (Santa Cruz Biotechnology, USA) for detection of synaptobrevin (specific substrate of the TeNT). Anti-mouse and anti-rabbit immunoglobulin-alkaline phosphatase (AP) conjugates were used as the respective secondary antibodies. The antigen-antibody reactive bands were then revealed by using BCIP/NBT chromogenic substrate.

Preparation of recombinant synaptobrevin (rSyb2)

Recombinant plasmid, pET-20b(+), carrying human synaptobrevin (rSyb2) was a kind gift from Professor Dr. Thomas Binz, Institut für Biochemie, Medizinische Hochschule Hannover, Germany. BL21 (DE3) pLysS *E. coli* cells were used as protein expression host for the recombinant synaptobrevin-pET-20b(+) vector. The transformed BL21 (DE3) pLysS *E. coli* bacteria were grown under IPTG induction. The bacterial cells were harvested and the rSyb2 was prepared and purified from the bacterial lysate by using a Ni-NTA agarose (Qiagen, Germany). The rSyb2 was verified by WB using rabbit anti-human VAMP1/2/3 as a detection reagent and goat anti-human immunoglobulin-AP and BCIP/NBT substrate as revelation reagents.

Tetanus toxin binding to human neuronal cell surface receptor and inhibition of the binding by TT-specific HuScFv

Human neuroblastoma cells of the SK-N-SH line were induced to express the surface receptors for

TeNT by using retinoic acid.[19] The cells were grown in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% penicillin/streptomycin in 5% CO_2 incubator at 37°C. Aliquots of 5 x 10^5 cells were placed in individual wells of a 24-well tissue culture plate and the cells in each well were pulsed with 10 μM retinoic acid (Sigma, USA). After 4 days, the differentiated cell monolayers were rinsed twice with PBS and fixed with 4% paraformaldehyde at 25°C for 20 minutes. TeNT (100 μl of 2.5 μg/ml binding buffer) was added to the cells and the plate was kept at 25°C overnight. After washing thoroughly, the TeNT bound to the cellular receptor was detected by using horse anti-tetanus toxin polyclonal antibody, mouse anti-horse immunoglobulin-AP conjugate and BCIP/NBT substrate, respectively. The results were observed by measuring OD of the content in each well at absorbance (*A*) 405 nm (A_{405nm}) against negative control (OD of content of well containing PBS instead of TeNT) using a Multiskan *EX*®, Labsystem ELISA reader. % TeNT binding to cells was (OD of TeNT + HuScFv ÷ OD of TeNT + buffer) x 100.

For determining the HuScFv mediated inhibition of the TeNT binding to the cell surface receptor, the TeNT (0.25 □ g) was pre-incubated with affinity purified HuScFv (4.8 □ g) (the molecular ratio of TeNT:HuScFv was 1:100) in binding buffer (0.25 mM sucrose, 20 mM Tris, 30 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25% BSA, pH 7.0) at 37°C before adding the mixture into the retinoic acid pulsed neuroblastoma cell monolayers. Cells added with TeNT mixed with the binding buffer (without HuScFv) served as negative inhibition control. The percentages of HuScFv mediated inhibition of cellular binding of the TeNT were calculated. % binding inhibition was 100 - % TeNt binding to cells.

HuScFv mediated-inhibition of synaptobrevin cleavage by tetanus toxin

Assay for zinc metalloprotease activity of TeNT was set up. TeNT was reduced by incubating with 10 mM DTT at 37°C for 30 minutes in order to expose its enzymatic active site. The reduced toxin (2 μg) was incubated at 37°C with 38.4 μg HuScFv from individual *huscfv*-phagemid transformed HB2151 *E. coli* clones (toxin:HuScFv ratio was 1:100) for one hour. A mixture of reduced TeNT and binding buffer instead of HuScFv served as negative protease inhibition control. Recombinant Syb2 (1.0 μg) was added to each TeNT + HuScFv mixture and the control and the preparation was kept at 37°C overnight. Each reaction mixture was then precipitated with trichloroacetic acid. The precipitate was dissolved in 10 μl of Laemmli buffer and analyzed by SDS-PAGE. The presence of intact rSyb2 band was revealed by immunoblotting by probing the membrane with rabbit polyclonal anti-VAMP1/2/3 (anti-Syb1/2/3), goat anti-rabbit immunoglobulin-AP and BCIP substrate, respectively.

RESULTS

Purified TT was successfully recovered from the commercialized adjuvanted tetanus toxoid vaccine after centrifugation and dialysis to remove the alum adjuvant and salts. The preparation was subjected to SDS-PAGE to reveal the correct protein size which was ~150 kDa (Fig. 1). The purified TT was used as antigen in a single-round phage biopanning to select phage clones that bound to the TT from the human antibody phage display library. From 25 randomly selected phagemid transformed *E. coli* derived from transfecting the competent HB2151 *E. coli* with the TT bound phages, lysates of 16 of the 25 clones grown under IPTG induction

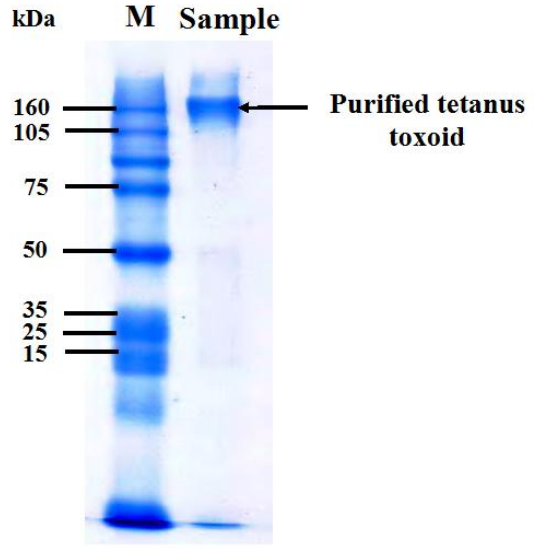

Fig. 1 SDS-PAGE pattern of tetanus toxoid purified from the alum adjuvanted tetanus toxoid vaccine after Coomassie Brilliant Blue R-250 staining (arrow).

(64%) gave significant indirect ELISA signal in binding to TT, *i.e.*, OD at A_{405nm} higher than the lysate of the original HB2151 (background binding of the *E. coli* lysate) and more than two times higher than the OD of BSA which was used as the negative antigen control (Fig. 2). From the 16 indirect ELISA positive clones, 14 clones (54%), *i.e.*, clones 1-3, 7-8, 10-13, 17, 19-23 and 25, revealed the *huscfv* sequence after amplification using the *R1* and *R2* as PCR primers (Fig. 3). Among the 14 clones, 7 clones (28%), *i.e.*, no. 1, 3, 8, 11-13 and 23, could produce HuScFv detectable by mouse anti E-tag monoclonal antibody in Western blot analysis (Fig. 4).

HuScFv from 4 randomly selected *huscfv*-positive clones (no. 1, 3, 12 and 13) were purified by using DEAE Sepharose resin and tested for binding to TT by dot-ELISA. HuScFv of all clones gave positive dot-ELISA (Fig. 5). The *huscfv* sequences of these 4 clones showed different DNA banding patterns after digesting with *Mva*I (Fig. 6).

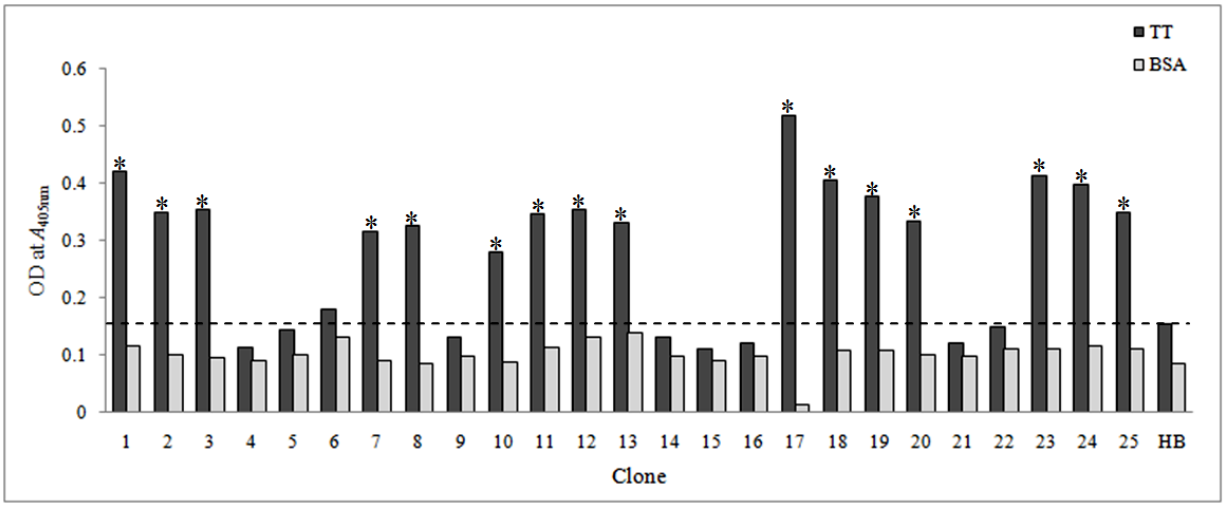

Fig. 2 Results of indirect ELISA for determining the binding of HuScFv in lysates of 25 randomly selected *huscfv*-phagemid transformed HB2151 *E. coli* clones that could recovered from phage bio-panning with tetanus toxoid. There were 16 indirect ELISA positive clones, *i.e.*, clones no. 1-3, 7, 8, 10-13, 17-20 and 23-25 which their HuScFv gave the ELISA signal (OD at A_{405nm}) above the lysate of the original HB2151 *E. coli* (background binding) and more than two times above the OD at A_{405nm} that the same HuScFv bound to BSA (antigen control).

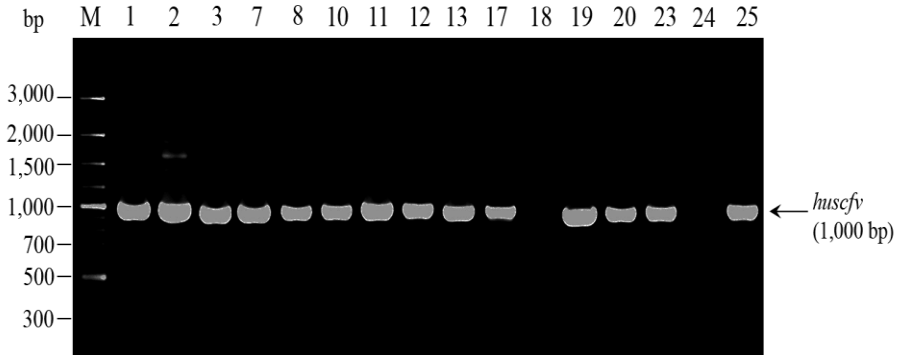

Fig. 3 Results of PCR for detection of *huscfv* in the 16 selected phagemid transformed HB2151 *E. coli* clones. Amplicons of *huscfv* could be detected from 14 clones, *i.e.*, clones 1-3, 7, 8, 10-13, 17, 19-23 and 24. Lane M, Standard DNA ladder. Numbers at the left are DNA band sizes in bp.

Inhibition of TeNT binding to receptor on retinoic acid pulsed human neuroblastoma cells by the soluble HuScFv of clones no. 1, 3, 12 and 13 were determined. It was found that in the presence of HuScFv of clones 1, 3, 12 and 13 (the TeNT:HuScFv molar ratio of 1:100), the percent binding of the TeNT was reduced to 76, 46, 52 and 36%, respectively, when compared to the binding of TeNT in buffer (absence of HuScFv) which was regarded as 100% binding (Fig. 7). Thus, the binding inhibitory activity of HuScFv, in falling order of magnitude, was of 64, 54, 48 and 24% for HuScFv of clones no. 13, 3, 12 and 1, respectively.

Inhibition of the TeNT protease activity by HuScFv from individual phagemid transformed HB2151 *E. coli* clones was also investigated by incubating HuScFv from each clone with TeNT prior to adding to the rSyb2 substrate. The results of Western blot analysis of the TeNT endopeptidase neutralization assay (band shift assay) are shown in Fig. 8. The intact rSyb2 is seen as a 12.8 kDa band after probing with anti-human VAMP1/2/3 (lane 1). Mixture of TeNT and rSyb2 yielded rSyb2 digestive product seen as a band at 10.1 kDa (lane 2). Preincubation of the TeNT with HuScFv from transformed *E. coli* clone no. 3 readily abrogated the proteolytic activity of the TeNT and the band of 12.8 kDa intact rSyb2 is revealed (lane 4). On contrary, HuScFv from the other *E. coli* transformants, *i.e.*, clones no. 1, 12 and 13, did not have the protease inhibitory activity and the rSyb2 substrate was digested to yield the 10.1 kDa bands (lanes 3, 5 and 6, respectively).

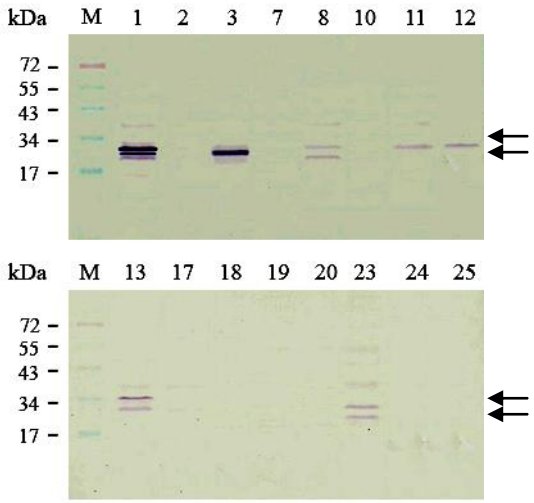

Fig. 4 Western blot analysis for detecting HuScFv in lysates of *huscfv*-positive HB2151 *E. coli* clones. Clones 1, 3, 8, 11-13 and 23 produced HuScFv shown as single or doublet bands at ~25-35 kDa (arrows). Lanes M, Standard protein marker. Numbers at the left of both blocks are sizes of proteins in kDa.

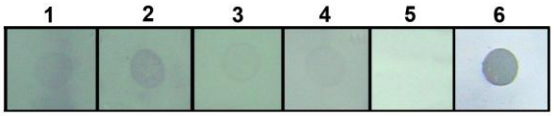

Fig. 5 Dot-ELISA results of the HuScFv of selected transformed HB2151 *E. coli* clones. Squares 1-4, HuScFv prepared from transformed HB2151 *E. coli* clones no. 1, 3, 12 and 13. Square 5, whole cell lysate of HB2151 *E. coli* without phagemids (negative control). Square 6, horse anti-tetanus toxin antibody (positive control)

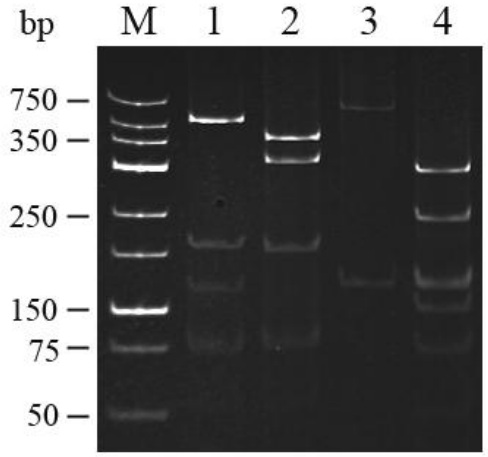

Fig. 6 DNA banding patterns for determining RFLP of *Mva*I-cut *huscfv* amplified from *huscfv*-pCANTABE5 phagemids transformed HB2151 *E. coli* clones no. 1, 3, 12 and 13. The *huscfv* sequences of the 4 clones revealed completely different restriction DNA banding patterns (lanes 1-4). Lane M, 100 bp standard DNA ladder. Numbers at the left are DNA band sizes in bp.

DISCUSSION

To date, antidote of the zinc metalloprotease activity of the tetanus neurotoxin light chain is not yet available. Remedy for human tetanus has been the same ever since early 1900s by passively giving the animal derived anti-tetanus serum/polyclonal immune immunoglobulins (PAb) together with supportive measures, such as an artificial respirator to the patient.[20] Several obstacles have been faced both in production and use of the therapeutic immune serum from animal. Major ones include the adverse reactions due to the foreignness of the heterologous proteins to the recipient immune system, the limitation of the supply of the immune serum and the batch-to-batch variation of the antitoxin potency.[10-11] On the basis of therapeutic mechanism, the therapeutic antibody blocks binding of the TeNT heavy chain (receptor binding domain) to the nerve cell receptors and prevent cellular entry but fails to inhibit the zinc metalloprotease activity of the intracellular light chain *per se*.[21] Extracellular TeNT does not expose its active enzymatic pocket.[22] Moreover, there has been a notion that large size antibody (intact IgG, 150 kDa) cannot insert its paratope into the active groove of enzymes.[23] Thus, even though the therapeutic immune serum that was raised against the tetanus toxoid (TT) may contain also the antibody that binds to epitopes of the TeNT light chain, they are unlikely to neutralize the zinc metalloprotease activity of the protein.

In this study, fully human single chain antibody fragments (HuScFv, ~27-35 kDa; ~5 times smaller than IgG) that bound specifically to the TT and blocks the biological functions of the native TeNT, *i.e.*, receptor binding and protease activity, were produced by a phage display technology. The *in vitro* production of the engineered-, small molecular sized-antibody bypassed the requirements of immunogen preparation (toxoiding of the TeNT), prolonged immunization process, *in vivo* immune regulation of the immunized animals, requirements for animal husbandry, and risk to zoonosis. The HuScFv are likely to be non-immunogenic in the human recipient and thus should not bring about any adverse reaction. HuScFv produced by a particular transformed cell should have stable target binding activity and affinity and free from a batch-to-batch variation. They should be produced and purified at the desired

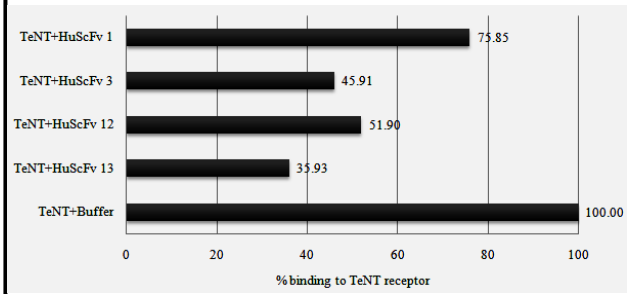

Fig . 7 Percent binding of HuScFv treated-and non-treated-TeNT to receptors on human neuroblatoma cells. HuScFv of clones no. 1, 3, 12 and 13 could reduce the TeNT binding to the receptors on the human neuroblastoma cells by ~76, 46, 52 and 36 %, respectively; thus the percentages of HuScFv mediated inhibition of the TeNT binding were 24, 54, 48 and 64%, respectively.

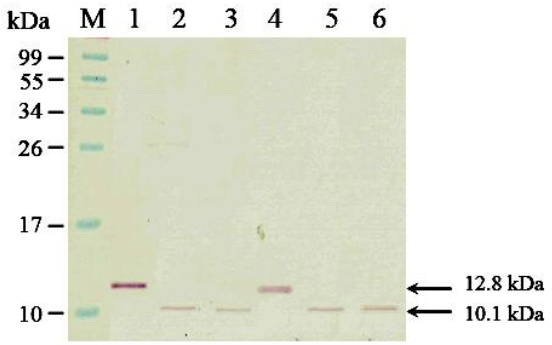

Fig. 8 Results of Western blot analysis for determining HuScFv mediated inhibition of rSyb2 cleavage by TeNT. SDS-PAGE separated reaction mixtures were probed with rabbit polyclonal anti-VAMP1/2/3 (anti-Syb1/2/3) in the Western blotting; the immune complexes were revealed by using goat anti-rabbit immunoglobulin-AP and BCIP substrate, respectively. Lane 1, Intact rSyb2 (12.8 kDa) in buffer (upper arrow); Lane 2, rSyb2 added with TeNT + buffer (the rSyb2 was digested by TeNT yielding a digestive product at ~10.1 kDa) (lower arrow); Lanes 3-6, rSyb2 added with a mixture of TeNT + HuScFv of clones no. 1, 3, 12 and 13, respectively. Intact rSyb2 band is shown in lane 4 indicating that HuScFv of clone no. 3 could readily inhibit the protease activity of the TeNT. HuScFv of clones no. 1, 12 and 13 did not have the TeNT protease inhibitory activity. Numbers at the left are protein masses in kDa.

speed and quantity in an *in vitro* cell expression system and a contemporarily high throughput purification process.

In this study, the TT used as an antigen in the phage bio-panning process to select phage clones that display TT bound HuScFv from a human antibody phage display library constructed in our laboratory, was conveniently purified from the commercially available tetanus vaccine. The same human antibody phage display library has been used successfully in producing HuScFv to long α-neurotoxin of Thai cobra (*Naja kauthia*) venom[14] as well as HuScFv that neutralize biological activities of various influenza A virus proteins, *i.e.*, basic and acidic polymerase proteins (PB1, PB2 and PA),[18] H5 hemagglutinin[24] and matrix protein M1.[15] These successes verified the adequate diversity of the HuScFv repertoire of the library. A single round phage bio-panning[14,15,18,24,25] was used with success also in our experiment. After the panning, several colonies of *E. coli* transformants appeared on the selective agar plate. We randomly picked only 25 transformants and kept the rest as the transformant stock from which more TeNT specific HuScFv clones can be screened, if needed. Among the 25 selected phagemid transformed *E. coli* clones, lysates of IPTG induced 16 clones (64%) gave significant indirect ELISA signal to TT above BSA which was used as the non-related (control) antigen. Nevertheless, *huscfv* sequences could be amplified from only 14 of these 16 clones (56%) indicating that the other two clones might have lost the recombinant plasmids. The single round panning offered us a high percentage of *huscfv*-positive clones in contrast to the repeated bio-panning described by other investigators which relatively high percentage of phage clones without *huscfv* (non-specific phages) were obtained (personal observation). HuScFv of 4 randomly selected transformed clones bound specifically to the TT by indirect and dot-ELISAs and also mediated varying degrees of inhibition of TeNT binding to the retinoic acid pulsed human neuroblastoma cells. HuScFv of clone no. 13 exerted the highest TeNT binding inhibition (64%). Complete TeNT binding inhibition should be achieved by increasing the amount of the respective HuScFv. The fifty percent effective dose (ED_{50}) of the HuScFv should be studied *in vivo*, such as in a mouse model. The results obtained from the *ex vivo* TeNT binding inhibition assay imply that either the HuScFv bind directly to the receptor binding motif or merely masking the receptor recognition site by steric hindrance. The finding that *huscfv* sequences of the 4 *E. coli* transformants had completely different RFLP implies that either the HuScFv are specific to different epitopes or merely they have different binding affinity to the same epitope.

The enzymatic groove of holo-TeNT is masked. The enzymatic active site becomes unmasked after the TeNT light chain exits from endosome into the neuronal cell cytoplasm. Thus, in our *in vitro* experiment for determining the HuScFv mediated inhibition of the TeNT protease activity, the commercialized holo-TeNT had to be treated with DTT in order to expose the enzymatic active site.[22] Among HuScFv of the 4 clones with inhibitory activity to TeNT receptor binding, HuScFv of one clone (no. 3) also inhibited the protease activity of the DTT reduced-TeNT. Possibility exists that epitope of HuScFv of clone no. 3 might be located at or in the immediate vicinity of the TeNT enzymatic groove on the toxin light chain and directly blocked accessibility of the enzyme to its substrate. Binding of the HuScFv to the epitope on the toxin light chain might concurrently mediate a steric hindrance of the receptor binding motif of the toxin heavy chain. Alternatively, epitope of the HuScFv of clone no. 3 might be on the heavy chain and HuScFv binding inhibited receptor binding and in the effect sterically interferes with the enzyme-substrate interaction. If this were the case, HuScFv of clone no. 3 should not be able to block the enzymatic activity of the cytoplasmic TeNT light chain *in vivo*. In all instances, epitopes of the HuScFv of the 4 clones should be sought in order to pinpoint the HuScFv molecular mechanism(s). The TT/TeNT binding affinity of the HuScFv and the synergistic effect of the HuScFv cocktail(s) on combating the native TeNT activities should be studied. Moreover, the HuScFv with TeNT protease inhibitory activity should be developed further into a non-toxic cell penetrating antibody (transbody), *e.g.* by linking with a cell penetrating peptide. Alternatively, the HuScFv may be delivered intracellularly by entrapping into a proper cell delivery vehicle such as liposome. The *in vivo* innocuity and kinetics of the antibody preparation(s) must also be studied. Definitely, there are, as yet, several milestones of research and development towards a real application of

the TeNT specific HuScFv in human. For the time being, however, we envisage that a cocktail of HuScFv and/or their cell penetrating version that bind to different TeNT epitopes and effectively combats the native TeNT activities should be a better alternative or a sole surrogate of the animal derived immunoglobulins in treatment of human tetanus.

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