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Original Article

Neutralization of Lethal Potency of Tetanus Toxin using Phage Display Produced scFv Antibody

Ehsan Khalili[1], Ebrahim Abbasi[2], Mahdi Aminian[1*]

1. Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
2. Department of Bacterial Vaccines, Razi Vaccine and Serum Research Institute, Karaj, Iran.

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Abstract

Background and Aim: Phage display technology provides a new approach for making human antibody fragments that could be applicable in passive immune therapy. We applied the use of this technology to make human single-chain variable fragments (scFvs) specific for tetanus toxin. Tetanus toxin is a neurotoxin constituted by the association of two subunits, mediates its lethal action by blocking neuromuscular vesicle docking.
Methods: We previously found that six Human scFv clones inhibit toxin binding to ganglioside GT1b. This is the final report of human tetanus scFvs (scFv 8 and scFv 13) isolated from an immunized library of more than 106 scFv clones with in vivo neutralizing activity.
Results: Only scFv 13 can reduce the in vivo toxicity induced by tetanus toxin. Also, scFv 8 has a weak capability of reducing the in vivo toxicity of the toxin.
Conclusion: These selected ScFvs can be considered as a possible option to substitute the human tetanus immunoglobulin (HTIG) which is extensively current immunotherapy for tetanus patients. Taken together, our results suggest that the use of human tetanus scFvs may lead to a less aggressive passive immune therapy against tetanus.

Keywords: Tetanus; Phage Display; scFv; Antibody.

[*]**Corresponding Author:** Mahdi Aminian; **Email:** amminian@tums.ac.ir

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Introduction

Tetanus has remained an issue in developing countries (1). Tetanus toxin is a protein released by the bacterium Clostridium tetani that cause the disease symptoms(2). The tetanus neurotoxin (TeNT) is produced as a 150 kDa single polypeptide that is posted translationally cleaved to produce a 50 kDa light chain joined by a disulfide bond to a 100 kDa heavy chain (2). The heavy chain can be cleaved into two fragments (H_N and H_C) with distinct functions. It has been proven that the HC fragment is involved in binding to sensitive cells and subsequent internalization into vesicles. The HN fragment plays an important role in the translocation of the L-chain across the vesicular membrane (3). It has been proposed that the light chain reduces the secretion of the inhibitory neurotransmitter (glycine and GABA) from the inhibitory interneurons into the synaptic cleft by undergoing a retrograde transport via the nerve axon to the spinal cord and, thereby resulting in a spastic paralysis (2).

Current management for tetanus includes TT-containing vaccine and human tetanus immunoglobulin (HTIG) as immunotherapy (4-6). There are numerous limitations of the human-derived immunoglobulin (7-10). Progress on recombinant antibody production has introduced new tools in the fields of therapeutic applications and provided an alternative to the hybridoma technology (11-14).

Applying smaller antibody fragments have advantages over whole immunoglobulins for some clinical applications, such as toxin identification and detoxification, good penetration of solid tumors, and rapid clearance (15-17). Phage display has introduced a useful means to develop a

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powerful method for the screening of libraries containing different peptides or proteins such as antibody fragments (11, 18-20). Currently, several therapeutic antibodies derived from phage display libraries are under clinical development (21-23).

In this study, a previously contrasted human single-chain fragment variable (HuscFv) phage library against anti-tetanus was assessed in mice model to find specific HuscFv antibodies against tetanus heavy chain(24). HuscFv phage was selected against TeNT and evaluated in vivo for its ability to neutralize the tetanus toxin. Now, we report the in vivo neutralizing potency of isolated scFvs specific for tetanus toxin from the large immunized phage display library described previously (24). After three rounds of panning, 15 scFv-phage clones displaying the desired specificity were obtained. High-affinity tetanus toxin neutralizing scFv maybe act as a therapeutic agent for alleviating the symptoms of tetanus toxin in infected individuals. We have constructed a panel of tetanus toxin-neutralizing antibodies, including single-chain variable fragments (scFvs). The engendered panel of anti-tetanus scFvs compete with the cellular receptor for tetanus binding. Protection against tetanus toxin challenge in a mice model correlated strongly with affinity, with the highest-affinity antibody.

Methods

Engineering and Construction of phage Antibody library

For this research, we applied the cloning and phage display system of previously constructed, which was quite satisfactory (24). To produce phage-scFv particles, 50 mL of E. coli TG1 transformants were cultured in 2xYT containing 100 mg/mL ampicillin and 1% glucose, shaking at 37ºC. At a culture density of OD600nm=0.5, 10^{12} pfu of M13KO7 helper phage were added to the bacteria and left at 37ºC with no shaking for 30 min followed by gentle shaking at 200 rpm for a further 30 min at 37C. The culture was then centrifuged and cell pellets were re-suspended in 50 mL of 2xTY containing 50 mg/mL kanamycin and 100 mg/mL ampicillin and cultured overnight at 30ºC with shaking at 250 rpm. The culture supernatant was centrifuged and 1:5 volume of ice-cold 20% PEG-6000 containing 2.5 M NaCl was added and incubated on ice for at least 2 h to precipitate the phage particles, which were collected by centrifugation at 20,000 g for 2 h at 4ºC. The phage was stored at -80ºC in PBS containing 10% glycerol. Infective titers of phages-scFv were determined using plaque count assay. TG1 grown to mid-log phase in 2xYT medium was infected for 30 min at 37ºC with a serial dilution of the phage. The samples were spread on 2xYT plates containing 50 mg/mL kanamycin and incubated overnight at 37ºC. The number of plaques was used to calculate the phage titers.

Screening of phage antibody library

Selection of phage particles displaying specific scFv fragments was performed on Immuno 96 MicroWellPlates (Nunc, Copenhagen, Denmark). TeNT used in this study was obtained from Razi Institute for Serums and Vaccines (Karaj, Iran). For the first panning round, 10 mg TeNT per well in 100 mL PBS were coated in the microplates overnight at 4ºC. For more stringent conditions in the second and third rounds of panning 1 and 0.1 mg TeNT per well were coated respectively. Following blocking with 5% (w/v) skim milk powder in PBS (5% MPBS), a library containing 1012 phage particles was added and the microplate was incubated for 2 h at room temperature. Non-bound phages were eliminated by washing 15 times with PBS containing 0.1% Tween-20 (PBST), followed by 15 times washing with PBS. The bound phages were eluted by incubation with 50 mL of 1 mg/mL trypsin for 15 min. The second elution was followed by adding 50 mL of 50 mM glycine-HCl (pH 2.2) for 15 min at room temperature. Eluted phages of each step were used to infect exponentially growth of E. coli TG1 cells by incubating for 30 min at 37ºC. Infected cells were spread on 2xYT plate containing ampicillin (100 mg/mL) and glucose (1% w/v); the plate was incubated overnight at 37ºC. Individual phage-infected clones were picked and grown for the production of phagemid particles. The culture was rescued using M13KO7 helper phage (Invitrogen,

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Waltham, MA). Rescued phage particles were used to initiate another two subsequent rounds of selection using a similar procedure.

Production of soluble scFv antibodies

The selected scFv-phage was inoculated into the E. coli ER2738 non-suppressor strain. Transfected E. coli cells carrying the phagemid encoding the scFv antibody were grown in 2 mL of 2xYT medium containing ampicillin (100 mg/mL) and glucose (1% w/v) at 37°C overnight. The overnight culture was used to inoculate 250 mL glucose-free 2XYT medium at 37°C and 250 rpm until the OD600 nm reached 0.5. The scFv expression was induced by 1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) for 7 h at 30°C and 250 rpm. Cells were centrifuged and sonicated for scFv extraction. Purification of scFv fragments was performed using immobilized metal affinity chromatography (IMAC) under native conditions. All purified proteins were analyzed by reducing 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie R-250. Western blot analysis was used to confirm the presence of His-tagged proteins at the correct molecular weight by anti-His-tag monoclonal antibody.

Neutralization of the tetanus toxin effect by the soluble scFvs

To evaluate the effect of scFvs on the lethality induced by tetanus toxin, two S.C LD_{50} of Tetanus toxin (10 ng) were preincubated with various concentrations of each scFv and injected into 22 g BALB/c mice. The mortality was followed every 24 h during 14 days and the results are expressed as the rate of survival (hours) of mice 24 h after the Tetanus Toxin/ScFv injection.

Results

Engineering of anti-tetanus ScFv antibodies

The heavy-chain (VH) and light-chain (VL) genes of antibodies were isolated by RT-PCR. Overlap extension PCR was used to produce 750 bp scFv gene fragments, with a sequence encoding the (Gly4Ser) 4 linker inserted between the C termini of the light chain sequences and the N termini of the heavy-chain sequences. The scFv genes were fused to the pIII protein in the vector Pcomb3x for display in a filamentous bacteriophage (Figure 1), and clones expressing the active protein were identified by phage enzyme-linked immunosorbent assay (ELISA). After expression and preliminary characterization of the scFv antibodies, 15 clones were entered to further studies (Figure 2a and 2b).

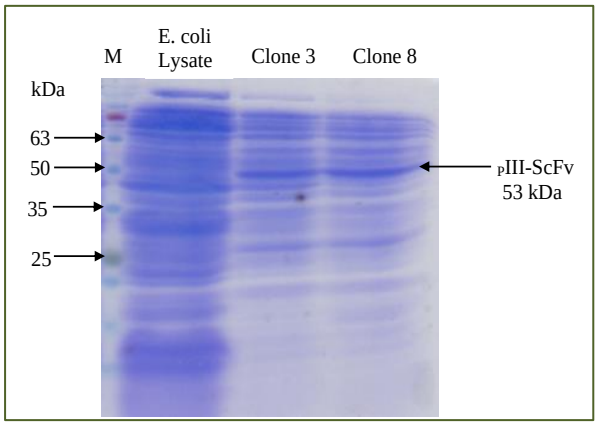

Figure 1. SDS-PAGE analysis for detecting pIII-scFv in lysates of scfv-positive ER2738 E. coli clones. Clones 3 and 8, produced pIII-scFv shown as a band at 53 kDa (arrows). Lane M, standard protein marker (value indicated at the left, in kilodaltons)

Neutralization of the lethal potency of Tetanus Toxin

Ten µg of the selected scFvs were incubated with 80 ng concentration of Tetanus toxin, and the mixture was then injected into mice. Some of the scFvs studied showed a neutralizing activity. However, the protection observed was different from one scFv to another. Indeed, scFvs 8 and 13 gave better protection than other scFvs (Table 1). The in vivo assay showed that the scFvs are specific for tetanus toxin.

Neutralization of the lethal potency of CTX

Different concentration of scFv1, scFv6, and scFv8 were incubated with a ®xed concentration of CTX, and the mixture was then injected to mice. All the scFvs studied show a neutralizing activity.

Tetanus Toxin (two s.c. LD50) was incubated with a fixed amount of each scFv (100 µg) as indicated in Materials and Methods. The mixture was injected into BALB/c mice and the mortality was followed every 24 hours. The results are expressed as the number of live mice 24h after the injection of the mixture.

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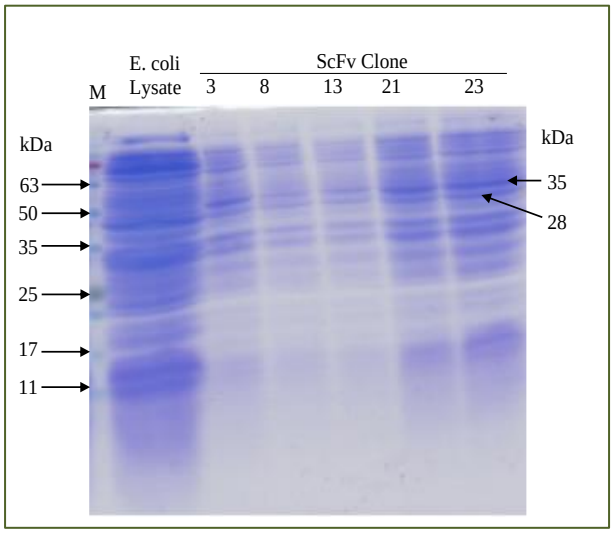

Figure 2 a. SDS-PAGE analysis for detecting scFv in lysates of scfv-positive ER2738 E. coli clones. Clones 3, 8, 13, 21, 23 and 23 produced scFv shown as double bands at *28–35 kDa (arrows). Lane M, standard protein marker (value indicated at the left, in kilodaltons).

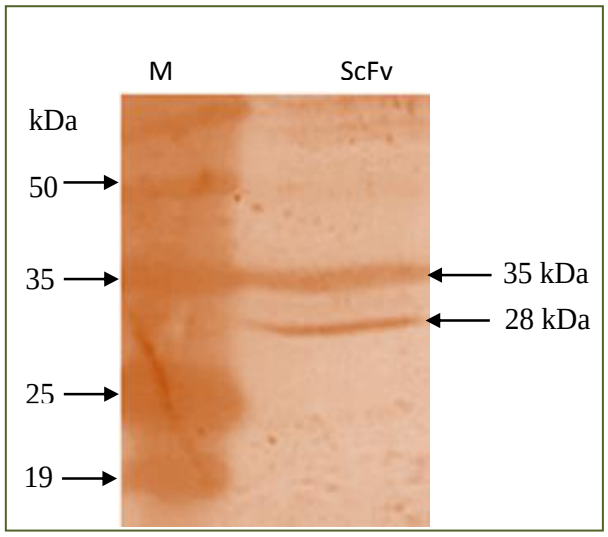

Figure 2b. Western blot analysis for detecting scFv in lysates of scfv-positive ER2738 E. coli clones detected with an anti-His tag antibody. Lane M, molecular mass marker (value indicated at the left, in kilodaltons). Lane ScFv, shown as double bands at 28–35 kDa (arrows).

Table 1. Effect of scFv on the lethality induced by Tetanus Toxin

Injected Mixture	Mice Numbers	Lifetime (Hour)
ScFv 3	3/3	24
ScFv 8	3/3	56
ScFv 12	2/2	30
ScFv 13	3/3	140
ScFv 14	2/2	24
ScFv 15	2/2	24
ScFv 16	2/2	24
ScFv 17	2/2	30
ScFv 18	2/2	30
ScFv 19	2/2	24
ScFv 20	2/2	30
ScFv 22	2/2	24
ScFv 23	2/2	36
ScFv 24	2/2	24
Tetanus Toxin	3/3	24
PBS	3/3	>200

Discussion

There is a growing demand for engineered proteins for therapeutic and diagnostic applications (25-28). Phage display technology has been successfully applied to produce antibody fragments that specifically bind to given antigens (29-32). In this study, we assessed the neutralizing ability of the previously constructed and screened phage antibody

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scFvs fragments library(24). To construct an antibody fragments library, the total mRNA was used from PBMCs of several donors who had received the booster tetanus vaccine. It has been shown that after boosting the affinity and specificity of antibodies are developed. We engineered the HuscFv format because the small size of scFv is well expressed in E. coli and typically high. (12). However, ScFv has rapid plasma clearance but exhibits high neutralizing ability.

The previous results obtained from the TeNT binding inhibition assay implied that the HuscFv binds directly to the receptor-binding motif (24). Therefore, it can be deduced that selected HuscFv against heavy chain can be considered a useful and efficient approach in tetanus management. By the current study, the in vivo neutralizing efficacy of selected scFvs was assessed. Further investigations should be applied to assess a cocktail composed of scfv antibodies that recognize different epitopes of TeNT. To obtain scFv with greater affinities, we need to utilize mutagenesis by using error-prone PCR. It seems that our selected HuscFv antibodies could have been considered as a candidate to enroll in the other complementary studies against TeNT or an emerging diagnostic tool.

Conclusion

These selected ScFvs can be considered as a possible option to substitute the human tetanus immunoglobulin (HTIG) which is extensively current immunotherapy for tetanus patients. Taken together, our results suggest that the use of human tetanus scFvs may lead to a less aggressive passive immune therapy against tetanus.

Conflict of Interest

The authors declared that they have no conflict of interest.

Acknowledgment

Not declared.

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Ethics

This study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1390.276).

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