RECOMBINANT HUMAN ANTIBODY FRAGMENT AGAINST TETANUS TOXOID PRODUCED BY PHAGE DISPLAY

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Phage display technology is a powerful *in vitro* method for the identification of specific monoclonal antibodies (antibody fragments) to an antigenic target and allows the rapid generation and selection of high affinity, fully human antibodies directed toward any disease target appropriate for antibody therapy. In the present study, we exploited the phage display technology for the selection of an antigen binding fragment (Fabs) toward tetanus toxoid using human naïve phage antibody library constructed from peripheral blood lymphocytes of naïve human donors. The phages displaying Fab were subjected to three rounds of bio-panning with tetanus toxoid as antigen on a solid phase. The high affinity antibody fragments were expressed in HB2151 strain of *Escherichia coli* and purified by immobilized metal affinity chromatography. The binding activity and specificity of the antibody fragment was established by its reactivity toward tetanus toxoid and non-reactivity toward other related toxins as determined by enzyme-linked immunosorbent assay and immunoblot analysis. The selected Fab fragment forming the antigen-binding complexes with the toxoid in flocculation assay indicates that the Fab may have a potential neutralizing ability toward antigen.

Keywords: Fabs, TT, flocculation, ELISA, phage

Introduction

Tetanus is a disease caused by the action of a powerful and potent neurotoxin produced by *Clostridium tetani* [1], both for humans and warm-blooded animals as well. *Clostridium tetani* is ubiquitous in nature, and humans usually get the infection via punctured wounds contaminated with the bacterial spores [2]. Infection begins when tetanus spores are introduced into damaged tissue. Tetanus is characterized by muscle rigidity and painful muscle spasms because of tetanus toxin's blockade of inhibitory neurons that normally oppose and modulate the action of excitatory motor neurons and results in death of the muscles [3, 4]. The toxicity of tetanus toxin is considered very high with an estimated human lethal dose of less than 2.5 ng per kg. Tetanus toxin is synthesized inside the bacterial cells as a single polypeptide chain of 150 kDa proteins [5]. Though tetanus is preventable by vaccination and post-exposure prophylaxis, the disease still remains as life-threatening, causing about thousands of death worldwide each year in places where the population is not extensively immunized [6].

Current treatment for tetanus involves the passive administration of anti-tetanus serum produced from immu-

nized animals. The use of polyclonal antibodies for treatment of tetanus has limitations because of allergic reaction and serum sickness developing in patients when treated with the antibody of the different species [7, 8]. The animal-derived antibody blocks the nerve cell entry of tetanus toxoid, and it does not neutralize the protease activity and cannot reverse the symptoms of tetanus [9].

To overcome the limitations of the traditional or conventional antibody preparations, recombinant antibodies have been widely used as a powerful alternative to conventional antibodies for the selection, screening, and production of custom-produced immunological reagents while preserving the advantages of monoclonal antibodies. Furthermore, the technique of displaying antibody fragment libraries on the surface of bacteriophage particles without apparent loss of the antibody specificity and affinity provides a powerful platform for the generation of human antibodies to a wide variety of infectious agents with a potential to serve directly as immune prophylactic, therapeutic, or diagnostic reagents.

Phage display technology has been utilized by several scientists for developing recombinant antibody fragments (Fab or scFv) of human, mouse, or other origins [10–13]. Antigen-specific recombinant antibodies can be easily iso-

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lated by biopanning of the phage library displaying antibody fragments fused with viral core protein III against antigen proteins [14–17].

In the present study, tetanus toxoid-specific human antibody fragments (HuFab) were affinity selected from a human naïve antibody library. The selected Fab fragment (F13) has shown specific binding activity with TT antigen in ELISA and also formed the antigen-binding complexes with tetanus toxoid in flocculation assay.

Materials and methods

Materials

Antigens and antibodies

The antigen of *C. tetani* was procured from the Human Biologicals Institute (HBI), Hyderabad. Antigens of *Clostridium septicum*, *Clostridium perfringens* Type D, *C. perfringens* Type B, and *Clostridium sordelli* were obtained from the Molecular Biology Laboratory, Indian Immunologicals Limited (IIL), Hyderabad. The Anti-M13 HRP conjugate was purchased from Sigma (St. Louis, MO). Anti-tetanus toxoid monoclonal antibody (2F8) of mouse origin was obtained from Hybridoma Laboratory, IIL. Tetanus toxoid immunized polyclonal horse serum was provided by HBI, Hyderabad. Human Naïve Fab antibody library was obtained from the National Institute of Health, USA. Anti-human IgG (Fab specific) HRPO was purchased from Sigma (St. Louis, MO).

Bacterial strains, vectors and chemicals

M13K07 helper phage for recombinant phage production was purchased from Invitrogen (Carlsbad, USA). The *Escherichia coli* strain TG1 used for the propagation of phagemid vector and the bacterial strain (*E. coli* HB2151) used for protein over expression were purchased from Stratagene (USA). All molecular biology reagents were purchased from Invitrogen (Carlsbad, USA). The plasmid isolation kit and Ni-NTA agarose used for the purification of His-tagged proteins were purchased from Qiagen (Hilden, Germany). *Sfi*I enzyme was purchased from Genei (India). Polymixin B sulphate, Imidazole and 3,3',5,5'-tetramethylbenzidine were purchased from Sigma (St. Louis, MO). All the other fine chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Methods

Propagation of human naïve Fab library

A single colony was picked from TG1 bacterial cell plate and grown in 9 ml of 2xYT medium at 37 °C until the optical density of the culture reaches 0.9 at 600 nm. Around

1 ml of phage Fab library (procured from NIH, USA) was infected to TG1 culture. The infection was carried out for 2 h at 37 °C and cells were harvested at 6000g for 10 min and resuspended in 10 ml of 2xYT medium containing 100 µg/ml of Ampicillin and 50 µg/ml of Kanamycin. Further, the culture was incubated at 30 °C for overnight. The overnight infected culture was harvested at 6000g for 10 min, and the phage particles were concentrated from the supernatant by polyethylene glycol precipitation (PEG 8000) [18]. The concentration of the infectious phage particles was determined by infecting log phase *E. coli* strain of TG1 cells with serially diluted phages and incubated for 10 min at 37 °C. The culture was plated onto 2xYT-Ampicillin Glucose agar plates and incubated overnight at 37 °C. Colony-forming units were counted the next day to determine phage library titers. The remaining phages were precipitated and resuspended in sterile phosphate-buffered saline (PBS) and filtered through 0.2-µm filter and stored at 4 °C. Only the freshly prepared phages were employed for biopanning in order to optimize the binding ability.

Biopanning of phage display library against tetanus toxoid

The propagated human naïve phage library was subjected to three rounds of biopanning for selection of Fab fragments using tetanus toxoid as antigen. Maxisorp immunotube (Nunc, Denmark) was coated overnight at 4 °C with tetanus toxoid (10 µg) diluted in 50 mM of sodium carbonate-bicarbonate buffer, pH 9.6. The tube was washed four times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and blocked with 3% skimmed milk in PBS-T (3% MPBS-T) and incubated at 37 °C for 2 h. The tube was washed as mentioned above and incubated with titrated phages at 37 °C for 2 h. Finally, the tube was washed 10 times with PBS-T and followed with PBS for 10 times. Log phase *E. coli* TG1 cells were added to the tubes and incubated at 37 °C to allow the infection by the bound phages. Infected culture was two-fold serially diluted and plated onto 2xYT-Ampicillin Glucose agar plates containing Ampicillin to determine the titer of phage [15]. The remaining culture was plated and incubated at 37 °C for overnight. The overnight grown cells were scraped and infected with helper phage M13K07 [19] and subjected to further rounds of panning. Three rounds of panning was performed with increasing selection stringency using higher concentrations of Tween-20 that ranged from 0.05% to 0.1% and also increased the number of washes from 10 to 20 times.

Selection of anti-tetanus toxoid-specific Fab fragments by phage ELISA

Analysis of phages for binding to tetanus toxoid by ELISA was performed on bacterial supernatants containing phages as previously described [20]. After three rounds of

biopanning, 480 individual colonies were inoculated into 100 µl of 2xYT-AG medium containing 0.2% glucose and 100 µg/ml Ampicillin and grown at 37 °C overnight with shaking at 200 rpm. An aliquot of 10 µl from the overnight culture was sub-cultured into 100 µl of 2xYT-AG media and incubated at 37 °C till the culture attained an OD₆₀₀ of 0.6 following which M13K07 helper phage of concentration 2.5×10^{10} pfu was added. Cultures were incubated with shaking at 37 °C for 2 h at 200 rpm and centrifuged at 4000g for 20 min. The bacterial pellet was resuspended in 2xYT containing 100 µg/ml Ampicillin, 50 µg/ml kanamycin and grown at 30 °C overnight with shaking at 200 rpm. Cells were pelleted, and the supernatant containing phages was collected for analysis in phage ELISA. Briefly, 96-well micro titer plate (Maxisorp, Nunc, Denmark) was coated overnight at 4 °C with tetanus toxoid diluted in 50 mM of sodium carbonate-bicarbonate buffer, pH 9.6. The plate was washed four times with PBS-T and blocked with 3% MPBS-T and incubated at 37 °C for 2 h. The plate was washed as mentioned above and incubated with phages containing 1% skim milk powder at 37 °C for 2 h. The plate was washed five times with PBS-T and further incubated with anti-M13 HRPO diluted in 1:5000 ratio (Sigma, USA) for 1 h at 37 °C. The plate was washed with PBS-T and detected with chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) by incubating at 37 °C for 10 min. The reaction was stopped by addition of 1.25M H₂SO₄, and the absorbance was measured at 450 nm using a micro titer plate reader (BIO-TEK, US). Similarly bound phages from each round of panning against tetanus toxoid were tested for specific binding to antigen by phage ELISA as the method mentioned above.

DNA sequencing

Clones selected by phage ELISA were analyzed further by nucleic acid sequencing. Phagemid DNA was purified using Qiagen Plasmid mini prep kit (Qiagen, Germany), according to the manufacturer's protocol and sequenced at the Eurofins Genomics India Ltd, India. The sequencing primers were 5'-ACCTATTGCCTACGGCAGCCG-3' for heavy chain and 5'-GTGTGGAATTGTGAGCGG-3' for light chain.

Expression and purification of recombinant F13 Fab fragment from periplasmic fraction

The identified positive clones from phage ELISA were transformed into *E. coli* HB2151 cells for expression and plated onto 2xYT agar plates containing 100 µg/ml Ampicillin and incubated for overnight at 37 °C. Individually selected clones were grown as previously described by Marks et al. [19]. The culture was induced by adding IPTG to a final concentration of 0.5 mM and incubated overnight at 30 °C with shaking at 250 rpm. The bacterial cells were subsequently harvested by centrifugation at 9000g for 15

min at 4 °C. The bacterial pellet was resuspended in 50 ml of PBS and supplemented with 0.5 million units (per liter) of polymixin B and incubated at room temperature for 30 min with rotation. The bacterial cells were centrifuged at 20,000g for 30 min at 4 °C, and the supernatant (periplasmic fraction) was subjected to Immobilized Metal Affinity Chromatography (IMAC) for the purification of recombinant F13.

Ni-NTA column (5 ml volume) was equilibrated with 10 column volumes of PBS-A, pH 7.5 (equilibration buffer). The supernatant was equilibrated with 0.3 M NaCl and 5 mM imidazole and incubated at room temperature for 10 min. The supernatant was loaded onto the column at a flow rate of 1 ml/min and washed with 20 column volumes of washing buffer (equilibration buffer with 30 mM imidazole, pH 7.5). Bound F13 Fab was eluted with 5 column volumes of elution buffer (equilibration buffer with 300 mM imidazole, pH 7.5). The eluted fractions were analyzed by SDS-PAGE and immunoblotting. Elution fractions containing the recombinant F13 Fab were pooled and dialyzed against PBS, and the protein concentration was estimated by bicinchoninic acid (BCA) method. The sample was stored at 20 °C till further analysis.

Analysis of F13 Fab expression by SDS-PAGE and Immunoblot analysis

The purified F13 was electrophoresed on a 12% SDS-PAGE. The resolved proteins were transferred to a polyvinylidene difluoride membrane (Hybond-C, Amersham Biosciences, USA) using semi-dry blot transfer apparatus (Bio-Rad, USA) following the manufacturer's instructions. The residual sites were blocked using 2% skimmed milk powder and probed with anti-human IgG (Fab-specific) HRPO 1:5000 (Sigma, St. Louis, USA). The blot was developed using 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, USA) and 0.03% hydrogen peroxide in PBS [21].

Analysis of F13 Fab binding activity with tetanus toxoid by immunoblot

Purified tetanus toxoid was fractionated by SDS-PAGE using a 10% gel under native condition. The resolved proteins were transferred onto a PVDF membrane (Hybond-C, GE Healthcare) and blocked with 2% skimmed milk powder (Difco, USA) in PBS for 1 h at room temperature. The blot was washed thrice with PBS containing 0.05% Tween-20 (PBS-T), followed by probing of the membrane with F13 Fab (200 µg/ml) for 1 h at room temperature. Tetanus toxoid-specific mouse mAb 2F8 was used as a positive control and *E. coli* lysate was used as a negative control. The immuno-reactivity of anti-tetanus toxoid antibody was detected by probing the blot with anti-human IgG (Fab-specific) HRPO at a dilution of 1:5000 followed by staining with the DAB substrate.

Antigen-binding activity of tetanus toxoid-specific Fab

Indirect ELISA was performed to evaluate the antigen-binding activity of purified F13 Fab. The F13 Fab was added as two-fold dilution to pre-coated well containing inactivated tetanus toxoid and incubated for 2 h at 37 °C. The *E. coli* lysate was served as a negative control. The wells were washed five times with PBST. Bound F13 Fab was probed with anti-human IgG (Fab-specific) HRPO and detected with TMB. The reaction was stopped by addition of 1.25M H₂SO₄, and the absorbance was measured at 450 nm using a microplate reader (BIO-TEK, US). The experiment was performed thrice and each time ELISA measurements were done in triplicate.

Immuno-capture ELISA for sensitivity of the F13 Fab against tetanus toxoid

A 96-well microtiter plate was coated with 2F8 mouse mAb (300 ng/well) in carbonate buffer (pH 9.6) and kept at 4 °C for overnight. The plate was blocked with skimmed milk powder (3%) and incubated with tetanus toxoid (1:2000 dilutions) at 37 °C for 1 h. After three washes with PBST, F13 Fab (2 µg) was added and serially diluted. The plate was incubated at 37 °C for 1 h. The Anti-Human IgG HRPO (Fab-specific) was added to all the wells. The plate was washed five times with PBS-T, and 100 µl of TMB substrate (Sigma, USA) was added. The plate was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 1.25 M H₂SO₄. The absorbance was measured at 450 nm using a microtiter plate reader (BIO-TEK, US).

Competitive ELISA

Competitive ELISA was performed to identify the specificity of the antibody binding fragment for tetanus toxoid. A Maxisorp microtiter plate was coated with 300 ng/well of tetanus toxoid antigen in duplicates and incubated overnight at 4 °C. The plate was washed thrice with PBS-T and blocked with 3% skimmed milk in PBS-T followed by washing with PBS-T to remove the excess skimmed milk. F13 Fab (2 µg/100 µl) was added by serial dilution into one set of wells, and tetanus toxoid-specific mouse mAb (2F8) was serially diluted in another set of wells. The plate was incubated at 37 °C for 1 h. *E. coli* lysate was used as a negative control. The mAb (2F8) and F13 Fab were diluted to 1:800 and 1:500 dilutions, respectively, and added to specific set of wells for competition. The plate was

incubated at 37 °C for 1 h and washed with PBS-T and dried by flicking. Anti-mouse HRPO conjugate (1:5000) and Anti-human IgG (Fab-specific) HRPO were added to a specific set of wells, and the plates were incubated at 37 °C for 1 h. The plate was washed five times with PBS-T and 100 µl of TMB substrate (Sigma, USA) was added. The reaction was stopped by addition of 100 µl of 1.25 M H₂SO₄ to each well, and absorbance was read at 450 nm using a microplate reader (BIO-TEK, USA).

Analysis of binding specificity of anti-tetanus toxoid F13 Fab toward unrelated antigens by ELISA

The binding specificity of the F13 toward tetanus toxoid and other related antigens was evaluated by testing its reactivity with *C. septicum*, *C. perfringens* Type D, *C. perfringens* Type B, *C. sordelli*, and *E. coli* lysate by Indirect ELISA as described by Sridevi et al. [20].

Flocculation assay

Limit of flocculation (Lf) values for samples containing tetanus toxoid were determined by flocculation test [22] using an in-house anti-tetanus serum which was calibrated against the 2nd International Standard for tetanus antitoxoid (NIBSC, UK) as a reference. The concentration of reference anti-tetanus serum is given as Lf-equivalent/ml [23]. The determination of sample Lf content was based on the fact that formation and subsequent precipitation of antigen-antibody complexes is the most prominent when the concentration of antigen and antibody is equal. Briefly, equal volumes of internal reference anti-serum in increasing concentration and TTn/TTd containing the sample and anti-tetanus toxoid Fab fragment were mixed and placed in a water bath preheated at 50 °C. The tubes were observed continuously, and the tube where the flocculation was the fastest was selected. The Lf value of the sample was calculated from the concentration of anti-serum in the tube.

Results

Biopanning against tetanus toxoid

The human naïve Fab library was screened to identify the specific Fabs against tetanus toxoid. The library was panned three times against immobilized tetanus toxoid an-

Table 1. Enrichment of tetanus toxoid-specific Fab clones in three rounds of biopanning

Input phages for each round of biopanning (pfu/ml)	Eluted phages (pfu/ml)		
	First round biopanning	Second round biopanning	Third round biopanning
5×10^9	1×10^3	2×10^4	2.5×10^6

Fig. 1. Determination of the biopanning efficiency by phage ELISA. Freshly prepared phages from each round of panning were added to an ELISA plate coated with tetanus toxoid and without coating. Bound phages were detected by using HRP-conjugated anti-M13 antibody

tigen bound to an Immuntube. It was observed that the Fab library exhibited an increase in phage output from round 1 to round 3 of selection (*Table 1*), suggesting that there was an enrichment of TT antigen-specific phage Fab. To further confirm that the Fab phage library was progressively enriched for tetanus toxoid antigen-specific binders, as indicated by polyclonal phage ELISA using the amplified phage from each round of panning, the average optical density (OD) ranged between 0.6 ± 0.032 and 1.6 ± 0.15 in the three different rounds of panning (*Fig. 1*). This demonstrated an increase in the signal in each round with

a strong specific response at the third round. There was no concomitant increase in signal as observed against helper phage, and phage antibody library.

Selection of tetanus toxoid antigen-specific Fab by phage ELISA

The Phage ELISA was performed to identify the positive clones that are displaying the specific Fab fragments against tetanus toxoid antigen. The eluted F13 Fab phages

Fig. 2. Isolation of Fabs from enriched phages after affinity selection. Individual clones of the third round selection were screened for binding activity in the ELISA

Fig. 3. Restriction digestion profile of clones digested with *Sfi*I

from round 3 were used to infect *E. coli* TG1 cells, and a total of 480 individual clones were tested for binding by phage ELISA against tetanus toxoid. Thirty-four positive clones were identified in the phage ELISA (Fig. 2), and the plasmids of these clones were analyzed for the presence of insert through restriction digestion. All the 34 clones showed the presence of 1.5 kb gene (Fig. 3, lanes 1–34). Twelve clones were chosen among the 34, based on the high OD values for sequence analysis.

Analysis of sequence

The selected twelve clones were sent for sequencing analysis. Phagemid DNA was purified according to the manufacturer's protocol and sequenced at Ocimum Biosolutions, India. The sequencing primers, Bomp and pelB, were used for variable light chain with constant region and variable heavy chain with constant region analysis, respectively. The entire sequence was submitted to the Interna-

Fig. 4. Consensus 4 amino acid sequence of anti-tetanus toxoid human Fabs containing VH and VL with constant regions. Clone 1 was selected for further study

Fig. 5. SDS-PAGE and Western blot analysis of Fab expression. Lane M, molecular mass marker; lane 1, eluted sample from Ni-NTA agarose column. (a) Sample was separated with 12% non-reducing SDS-PAGE, followed by coomassie blue staining (b) For Western blotting, eluted sample from Ni-NTA affinity column was transferred onto a PVDF membrane and probed with anti-human IgG Fab HRPO. The antibody was detected by using DAB substrate

Fig. 6. The reactivity of the Fab fragment with the tetanus toxoid in immuno blot analysis

tional ImmunoGeneTics information system (IMGT) [24] for sequence verification. Clones which showed without stop codons were taken for amino acid homology comparison. The sequence homology study revealed that four clones are showing 100% similarity in constant regions of heavy and light chains. High diversity was observed in the variable regions of heavy and light chains of all the four clones (Fig. 4). One specific clone (F13 Fab) was selected among the four clones based on the binding activity in phage ELISA. Further characterization of this clone

showed the presence of 650 bp light chain sequence with constant region and 708 bp heavy chain sequence with constant region.

Soluble expression of F13 Fab fragment

The positive F13 Fab clone was transformed into non-suppressor *E. coli* strain HB2151 cells for expression of soluble F13 Fab for further characterization. The F13 Fab fragment was expressed and purified as mentioned in Methods section. The purified protein showed a 46-kDa band on both SDS-PAGE and immunoblot (Fig. 5a, lane 1 and 5b).

Reactivity of antibody binding fragment with tetanus toxoid

Immunoblotting of the F13 Fab against the tetanus toxoid resolved in a 10% gel by non-reducing SDS-PAGE clearly indicated the binding of the Fab toward tetanus toxoid antigen at ~150 kDa (Fig. 6, lane 1). The anti-tetanus toxoid human mAb (2F8), which was used as a positive control, also showed a clear band at ~150 kDa (Fig. 6, lane 2), and no band was observed in the periplasmic fraction of *E. coli* HB2151 which was used as a negative control (Fig. 6, lane 3).

Fig. 7. Analysis of antigen-binding affinity of F13 Fab by Indirect ELISA to evaluate the activity of Fab toward tetanus toxoid

Analysis of F13 Fab binding activity of tetanus toxoid antigen by Indirect ELISA

The antigen-binding activity of the purified F13 Fab was analyzed by Indirect ELISA. Titration of F13 Fab against the tetanus toxoid revealed a concentration dependent reduction of the optical density values as shown in Fig. 7. The F13 Fab showed significant binding activity at high concentration (2 µg/100 µl) with the OD of ~1.0 and gradually reduced to ~0.1 with the decrease in concentration. The periplasmic fraction of HB2151 showed no binding activity with tetanus toxoid antigen which served as the negative control.

Immunocapture ELISA

Sandwich ELISA was performed to determine the specificity of F13 Fab toward tetanus toxoid. 2F8 Mouse mAb was coated as a capture antibody, and titration of tetanus toxoid antigen against a constant dilution of F13 Fab revealed a concentration-dependent reduction of the binding signal in the wells (Fig. 8). The *E. coli* lysate used as a negative control showed negligible binding activity with tetanus toxoid antigen in the ELISA plate. This indicated that F13 Fab is binding specifically with tetanus toxoid.

Fig. 8. Analysis of F13 Fab activity toward tetanus toxoid in sandwich ELISA

Fig. 9. Competitive ELISA using the F13 Fab and the mouse monoclonal antibody

Fig. 10. Specificity of F13 Fab toward various *Clostridium* species toxins

Competitive ELISA

Competitive ELISA was performed to determine the competition between antibody binding fragment (F13 Fab) and mouse mAb 2F8 for the binding site on tetanus toxoid. No competition could be seen when the constant amount of mouse mAb was allowed to compete with varying amount of the F13 Fab. And there was no competition observed in vice versa condition. Constant OD values (around ~1.0) in the dilution of both antibodies indicated that the F13 Fab

and mouse mAb were binding to different antigenic sites on tetanus toxoid. The constant OD values in negative control supported the above-mentioned results (Fig. 9).

Specificity of F13 Fab

Indirect ELISA was performed to determine the binding specificity of the F13 Fab toward toxins of other different *Clostridium* species. The F13 Fab clearly showed specific

Fig. 11. Neutralizing efficiency of F13 Fab toward tetanus toxoid by flocculation assay. Neutralization expressed in the limit of flocculation

binding activity against toxoid of *C. tetani* up to the OD of 1.0. However, the specificity of F13 Fab toward the toxins of other species like *C. septicum*, *C. perfringens* Type D, *C. perfringens* Type B, and *C. sordelli* was considerably low, and the OD was around 0.1. This indicated that the generated F13 Fab is species specific and could be used to detect tetanus toxoid without any cross-reactivity to other related antigens (Fig. 10).

Flocculation assay

The F13 was further analyzed for its neutralizing efficiency against tetanus toxoid through flocculation assay. The assay was performed as mentioned in Methods, and the limit of flocculation (Lf) for F13 Fab was calculated as 320 Lf/ml. The immunized horse polyclonal sera which served as positive control showed 350 Lf/ml, while the negative control showed no Lf value (Fig. 11). This clearly indicated that the F13 Fab is showing neutralization reaction against tetanus toxoid antigen.

Discussion

Tetanus is a fatal disease, which causes the high mortality rate between 40% and 78%, mainly due to respiratory failure [25, 26]. Most cases of tetanus result from small puncture wounds or lacerations which become contaminated with *C. tetani* spores that germinate and produce toxin leading to death of voluntary muscles. The development of fully humanized antibodies *in vitro* bypassing the animal utilization would have a potential use in therapeutics. Antibody-based therapeutics are attracting more attention in the post-genome era, in contrast to a reduction in rapid development of gene-based therapeutic modalities. In support to the antibody-based therapeutics, the advent of recent technologies has made human antibody screening easier for production and more cost-effective. Among the different technologies, the phage display antibody technology has been successfully applied in the development of antibody-based drugs. The human antibody sources have become a valuable tool for antibody engineering, screening of high quality and high binding antibodies from large and highly diversified antibody libraries [27, 28]. Naïve human libraries give a broad range of screening for different diseases because of their high diversity [29]. Naïve libraries have been used to identify antibody fragments of different antigen molecules till date [30].

In this article, we describe the identification of specific Fab expression, purification, and immunological characterization of a human antibody fragment followed by its application in neutralizing the tetanus toxoid in flocculation assay. We selected human monoclonal F13 Fab against tetanus toxoid from the human naïve library using 5×10^9 pfu/ml and found 85% of recombination rate. The selected F13 Fab fragment was expressed in *E. coli*

as periplasmic protein with the size of ~ 46 kDa soluble molecule. The F13 Fab was purified by immobilized metal affinity chromatography (IMAC). Purification of bacterial periplasmic fraction resulted in ~7 mg of purified F13 Fab/L of culture with a homogeneity of ~85%. The ease of purification reiterated the fact that expression of a functional recombinant antibody in bacteria offered many advantages over the maintenance of a hybridoma cell line, which included minimal batch-to-batch variation, ease of scale up, etc. at a reasonable cost.

In order to demonstrate the binding of F13 Fab to the tetanus toxoid, immuno capture ELISA was performed. The F13 Fab showed significant binding to the TT antigen in immune-capture ELISA. The F13 Fab showed specific binding activity toward *C. tetani* and not with other Clostridium toxoids. Earlier reports showed that phage displayed Fab which was assembled from the variable heavy chain and variable light chain of anti-TT monoclonal hybridoma cells has shown significant binding activity with TT antigen [31]. However, the F13 Fab was screened from the human naïve library and shows significant binding activity with the antigen. The human scFvs screened from the human antibody library against TT antigen inhibited the metalloprotease activity of TT antigen [28]. It is evident that the identified scFv can significantly bind to the TT antigen. Our study reports that F13 Fab molecule identified from the human naïve library has shown significant binding and specificity toward the TT antigen.

The F13 Fab has shown neutralization with the TT antigen in the flocculation assay. Though the flocculation limit was not adequate to the positive polyclonal sera, the efficiency of neutralization was significant. Since the TT antigen has multiple epitopes, we performed a competitive ELISA to identify the epitope binding site of commercially available mAb and F13 Fab. The results revealed that F13 Fab and mAb are not competing with each other to bind on single epitope. This clearly indicated that the F13 Fab and mAb are recognizing different epitope in the TT antigen.

Tetanus toxoid vaccination has recently been made mandatory for children and has been included as a part of the regular vaccine program [32]. During the process of vaccine development, it is essential to establish vaccine characteristics as good quality and protective levels of antigen in the batches [33]. Quantification of antigen is vital for blending of antigen in vaccine batches, and these quantifications are being done through the ELISA method [21, 27]. The F13 Fab would be utilized further in the development of an in-house ELISA format for quantification of tetanus toxoid antigen and in passive immunization.

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