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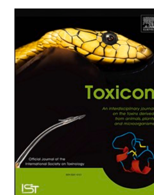

Neutralization of tetanus toxin by a novel chimeric monoclonal antibody

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ABSTRACT

Purpose: Tetanus is a life-threatening disease characterized by muscle spasm caused by neurotoxin of Clostridium tetani. Given the current passive immunotherapy of tetanus with human anti-toxin polyclonal antibodies (PAbs) and the limitations of such preparations, neutralizing monoclonal antibodies (MAbs), especially chimeric or human antibodies with reduced immunogenicity might be considered as an alternative source.
Methods: A mouse-human **c**himeric MAb, designated c-1F2C2, was generated and its binding specificities to various recombinant fragments of tetanus toxin, generated in *E. coli*, were determined. *In vivo* toxin neutralizing activity of c-1F2C2 was evaluated and compared with that of a commercially available human anti-toxin PAb in a mouse model. The possible mechanisms of toxin neutralizing activity of c-1F2C2 were investigated by assessing its inhibitory effects on toxin receptors binding, including GT1b ganglioside receptor and those expressed on PC12 cells.
Results: In vivo neutralizing assay showed that c-1F2C2 was able to protect mice against tetanus toxin with an estimated potency of 7.7 IU/mg comparing with 1.9 IU/mg of the commercial human anti-toxin PAb for 10 MLD toxin and 10 IU/mg versus 1.9 IU/mg of the PAb for 2.5 MLD toxin. c-1F2C2 recognized fragment C of the toxin, which is responsible for binding of the toxin to its receptor on neuronal cells. Accordingly, the chimeric MAb partially prevented the toxin from binding to its receptors on PC12 cells (37% inhibition).
Conclusion: The chimeric MAb c-1F2C2 displayed similar structural and functional characteristics compared to its murine counterpart and might be useful for passive immunotherapy of tetanus.

1. Introduction

Tetanus neurotoxin, produced by *Clostridium tetani,* is the second deadliest known toxin in the world (UF, 2014). The toxin degrades synaptobrevin, which mediates exocytosis of the inhibitory neurotransmitters, such as γ-aminobutyric acid and glycine from inhibitory neurons, resulting in the tetanic spasm which occurs in the disease (Link et al., 1992). The toxin consists of two chains, including heavy chain (HC) and light chain (LC). The heavy chain is composed of two functional fragments: fragment B, located at the N-terminal domain, which is involved in the LC entrance into the cytosol of neuronal cells, and fragment C at the C-terminal domain, contributing to the toxin binding to neuronal cells, which is required for the toxin entrance into the cells (Helting and Zwisler, 1977). It is well known that the C-terminal portion of fragment C (HCC) contributes to the neuronal binding. In addition, another sub-domain located at the N-terminal end of fragment C (HCN) includes the residues essential for proper folding and stability of fragment C (Emsley et al., 2000; Sinha et al., 2000). On the other hand, the LC (fragment A) implicates in degradation of synaptobrevin, thereby inhibiting the release of inhibitory neurotransmitters, leading to sustained spasms which occur in tetanus (Link et al., 1992).

Passive immunotherapy for tetanus is achieved by anti-tetanus toxin neutralizing polyclonal antibodies (PAbs) which are prepared from human hyper-immunized donors. Given the limitations of polyclonal immunoglobulin preparations, an alternative source of neutralizing antibodies should be considered to replace polyclonal immunoglobulins. Monoclonal antibodies (MAbs), directed against a single epitope of an antigen, serve as suitable substitutions for human anti-toxin PAbs (Pelfrene et al., 2019; Saylor et al., 2009; Wagner and Maynard, 2018). Murine MAbs, generated in mice, are recognized as foreign proteins by human immune system, leading to induction of human anti-mouse antibody (HAMA) response. HAMA response results in the elimination

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of MAbs by reticuloendothelial system. Chimeric MAbs, in which murine immunoglobulin variable regions are fused to human constant regions by recombinant DNA technology, reduce the HAMA response in humans (Abbas et al., 2017).

In the present study, a previously generated and characterized mouse MAb, designated 1F2C2, with *in vivo* neutralizing activity against tetanus toxin (Yousefi et al., 2014a), was converted into a mouse-human chimeric antibody and its structural and functional activities were investigated *in vitro* and *in vivo* and compared with those of a commercial human anti-toxin PAb.

2. Materials and methods

2.1. Construction of antibody variable region genes

To prepare the gene construct for the chimeric antibody, pBudCE4.1 vector containing c-1F2C2, variable regions of the heavy chain (VH) and light chain (VL) of the mouse MAb, 1F2C2, was amplified by polymerase chain reaction (PCR). The PCR reactions performed in 25 μl volume containing10× reaction buffer (2.5 μl), 50 mM $MgCl_2$ (1.2 μl), 10 mM dNTP (1.5 μl), Pfu DNA polymerase (0.2 μl, Vivantis, Malaysia), 10 pM forward and reverse primers (3 μl), 1:10 diluted complementary deoxynucleic acid (cDNA) (1 μl) and DNase, RNase free water (12.6 μl). The cDNA used in the PCR reaction was synthesized from the previously extracted hybridoma ribonucleic acid (RNA) (Yousefi et al., 2014a). Primer sequences are listed in Table 1. PCR conditions for amplification of the light and heavy variable regions were the same: an initial denaturation step of 3 min at 95 °C followed by 40 cycles, including a denaturation step at 94 °C for 30 s, an annealing step at 56 °C for 30 s and an extension step at 72 °C for 2 min followed by a final extension step at 72 °C for 10 min.

The amplified VH and VL genes were cloned into TA vector (Sinaclon, Tehran, Iran) and sequenced. After aligning the sequences in the ImMunoGeneTics (IMGT) website (2020), specific primers were designed to amplify the VH and VL regions from the beginning of the leader sequence to the end of the J segment and VH and VL regions were amplified by these primers. Primer sequences are listed in Table 2. These PCRs were performed in a reaction mixture containing 10× reaction buffer (2.5 μl), 50 mM $MgCl_2$ (0.6 μl), 10 mM dNTP (1 μl), Pfu DNA polymerase (0.2 μl), 10 pM forward and reverse primers (1 μl), 1:10 cDNA (1 μl) and DNase, RNase free water (17.7 μl). The PCR conditions were the same as those mentioned above. All materials and enzymes used for PCR were purchased from Vivantis company (Malaysia).

2.2. Amplification of human IgG1 and Igκ constant region genes

Constant regions of human gamma 1 heavy chain (Cγ1) and kappa light chain (Cκ) were amplified by PCR under conditions used for the amplification of VH and VL regions employing specific primers. The sequences of the primers are listed in Table 3.

Human Cγ1 and Cκ were joined to the mouse VH and VL regions, respectively, by splicing overlap extension PCR (SOEing PCR). This PCR was performed in two steps. In step one, the PCR reaction was carried out as follows: the reaction mixture (20 μl) consisted of 10× reaction buffer (2 μl), 50 mM $MgCl_2$ (0.6 μl), 10 mM dNTP (1 μl), Pfu DNA polymerase (0.2 μl), Cγ1 or Cκ gel extracted-PCR product (0.6 μl), VH or VL gel extracted-PCR product (1 μl) and DNase, RNase free water (14.6 μl). SOEing PCR condition was as follows: 3 min denaturation at 95 °C followed by 10 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 63 °C for 30 s and an extension step at 72 °C for 2 min followed by a final extension step at 72 °C for 10 min. In the second step, the reaction mixture containing 10× reaction buffer (0.5 μl), 10 pM forward and reverse primers (1 μl) and DNase, RNase free water (2.5 μl) was added to each tube. PCR condition was the same as the first step except for the number of cycles which was 35 cycles. Primer sequences are listed in Table 4. The amplified chimeric VH-Cγ1 and VL-Cκ were cloned into pBudCE4.1 vector and bacteria containing both VH-Cγ1 and VL-Cκ constructs were selected based on the colony PCR. All materials and enzymes were purchased from Vivantis company (Malaysia).

Table 1
Sequences of the degenerate primers used for amplification of VH and VL regions of mouse 1F2C2.

	Type of primer	Sequence (5′-3′)	Amplicon size
VH	Forward	CAGGTSMARCTGCAGSAGTCWGG	396 bp
	Reverse	AGGGGCCAGTGGATAGACAGATGG	
VL	Forward	GAHRTTSWGNTSACYCAGWCTCCA	366 bp
	Reverse	TGGTGGGAAGATGGATACAG	

The primers cover mouse variable regions from any immunoglobulin gene family.
VH: Variable region of mouse heavy chain, VL: Variable region of mouse light chain.

2.3. Stable transfection of c-1F2C2 in CHO cells and purification of the chimeric MAb

For expression of the chimeric antibody, pBudCE4.1 vector containing c-1F2C2 construct was transfected into eukaryotic adherent Chinese hamster ovary (CHO–K1) cells (Pasteur Institute of Iran, Iran) by lipofectamine transfection reagent (Invitrogen, USA) and stable clones producing c-1F2C2 were generated and cloned using Zeocin selection reagent (Thermo Fisher Scientific, USA) followed by limiting dilution assay.

Successful transfection was evaluated using an indirect enzyme-linked immunosorbent assay (ELISA). Briefly, tetanus toxoid (10 μg/ml) (Razi Vaccine and Serum Research Institute, Karaj, Iran) was coated on the wells of an ELISA plate (Maxisorp, Nunc, Denmark) and blocking was performed by 3% skim milk (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS)-Tween 0.05% (PBST) solution. Culture supernatant was collected after 48 h of transfection and different concentrations of human anti-toxin PAb which was used as positive control, were added to the wells. In the next step, appropriate dilution (1:2000) of horse radish peroxidase (HRP)-conjugated sheep anti-human antibody (Sina Biotech, Tehran, Iran) was added to the wells. The incubation temperature and time in all steps were 37 °C and 1.5 h. The washing step with PBST was carried out following each step for 3 times. Finally, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (PishtazTeb, Karaj, Iran) substrate solution was added to the wells, followed by addition of stop solution. The optical density (OD) of the samples was finally measured at 450 nm by an ELISA reader. Large scale culture of the final clone was carried out in a serum free culture medium (Inoclon, Tehran, Iran), the supernatants were collected and the antibody was purified using streptococcal protein G (SPG) column (GE Healthcare Life Sciences, Pittsburgh, USA). The purity and structure of the purified antibody were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions.

2.4. Evaluation of toxin binding by c-1F2C2

The specificity of c-1F2C2 for tetanus toxin was assessed by indirect ELISA and Western blot assays. For indirect ELISA, tetanus toxin (10 μg/ml) (Razi Vaccine and Serum Research Institute, Karaj, Iran) was coated on the wells on ELISA plate (Nunc). After blocking, various concentrations of the MAbs, including c-1F2C2, 1F2C2 and human or mouse anti-toxin PAb were added to the wells. The PAbs served as positive controls in the experiment. In the next step, appropriate dilutions (1:2000) of HRP-conjugated anti-human or mouse antibody, including sheep anti-human antibody and rabbit anti-mouse antibody (Sina Biotech) were added to the primary human and mouse antibodies, respectively. The next steps were the same as those used for the indirect ELISA with the toxoid. The incubation temperature and time in all steps were 37 °C

Table 2
Sequences of the specific primers used for amplification of VH and VL regions of mouse 1F2C2.

	Primer	Type of primer	Sequence (5′-3′)	Amplicon size
VH	SalI-F2C2-VH-S-5′	Forward	GTTTTGTTGTCGACGCCGCCACCATGGAAGTGTTGAGTCTGTTGTACCTGTTG	434 bp
	F2C2-JH–CH–AS-5′	Reverse	GGGCCCTTGGTGGAGGCTGCAGAGACAGTGAC	
VL	KpnI-F2C2-VK-S-5′	Forward	GTTTTGTTGGTACCGCCGCCACCATGGAGTCACAGATTCAG	397 bp
	F2C2-JK-CK-AS-5′	Reverse	GGTGCAGCCACAGTTCGTTTGATTTCCAGCTTGG	

The designed primers amplified VH or VL DNA sequence of mouse 1F2C2 starting from the leader sequence of variable genes to the end of J gene segment.
VH: Variable region of mouse heavy chain, VL: Variable region of mouse light chain.

Table 3
Sequences of the primers used for amplification of human Cγ1 and Cκ regions.

	Primer	Type of primer	Sequence (5′-3′)	Amplicon size
Cγ1	CH–S	Forward	GCCTCCACCAAGGGCCCATCGGTC	990 bp
	BamHI–CH–AS	Reverse	TTTTTTTTGGATCCTCATTTACCCGGAGACAGGGAGAGGCTCTT	
Cκ	Cκ-S	Forward	ACTGTGGCTGCACCATCTGTCTTCATCTTCCC	321 bp
	XhoI-Cκ-AS	Reverse	TTTTTTTTCTCGAGCTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGA	

Cκ: Constant region of kappa light chain, Cγ1: Constant region of gamma 1 heavy chain.

Table 4
Sequences of the primers used for amplification of chimeric mouse-human VH-Cγ1 and VL-Cκ regions in SOEing PCR.

	Primer	Type of primer	Sequence (5′-3′)	Amplicon size
VH-Cγ1	SalI-F2C2-VH-S-5′	Forward	GTTTTGTTGTCGACGCCGCCACCATGGAAGTGTTGAGTCTGTTGTACCTGTTG	1424 bp
	BamHI–CH–AS	Reverse	TTTTTTTTGGATCCTCATTTACCCGGAGACAGGGAGAGGCTCTT	
VL-Cκ	KpnI-F2C2-Vκ-S-5′	Forward	GTTTTGTTGGTACCGCCGCCACCATGGAGTCACAGATTCAG	718 bp
	XhoI-Cκto-AS	Reverse	TTTTTTTTCTCGAGCTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGA	

VH-Cγ1: Variable region of mouse heavy chain plus constant region of human gamma 1 heavy chain, VL-Cκ: Variable region of mouse light chain plus constant region of human kappa light chain.

and 1.5 h, respectively.

For Western blotting, 2.5 μg of tetanus toxin under non-reducing and reducing conditions, were loaded into the wells of a 12% SDS-PAGE gel and electrophoresed. The proteins were then transferred onto a nitrocellulose membrane (Schleicher & Schuell, Germany). The membrane was then blocked by 5% skim milk in PBST. The MAbs and PAbs (20 μg/ml) were added to the membrane followed by washing and addition of HRP-conjugated antibodies. The bands were then visualized by addition of enhanced chemiluminescence (ECL) substrate solution (GE healthcare life sciences) solution and recorded on an autoradiography film. All steps were carried out at room temperature for 1.5 h with shaking. The washing step with PBST was carried out for 5 times after each step.

Correct insertion of human Cγ1 and Cκ into c-1F2C2 was examined using an indirect ELISA. All steps in these ELISAs were the same as those used in the ELISA assessing reactivity of the MAbs to the toxin, except that an anti-human IgG MAb (Sina biotech) was coated on the ELISA plate wells and HRP-conjugated sheep anti-human antibody (Sina Biotech) andgoat anti-human kappa light chain antibody (Dako, Glostrup, Denmark) were used for detection of Cγ1 and Cκ, respectively.

2.5. *Determination of affinity constant of the chimeric MAb*

Affinity constants of c-1F2C2 and its murine counterpart were determined using an indirect ELISA, as previously described (Golsaz--Shirazi et al., 2017). Briefly, serially diluted concentrations of the MAbs, including 0.5, 0.25, 0.12, 0.06, 0.03, 0.015, 0.007 and 0.003 μg/ml and serially diluted concentrations of tetanus toxoid (1, 0.5 and 0.25 μg/ml) were used in this ELISA. Thereafter, sigmoidal curves were plotted using OD values of different antibody concentrations. The affinity constant was determined according to the following formula: $Ka = \frac{(n-1)}{2(n[Ab]-[Ab])}$, where $n = \frac{[Ag]}{[Ag]}$ and *[Ab]*represents the antibody concentration giving half maximal OD (OD 50) at the antigen concentration of *[Ag].[Ab]* represents the antibody concentration giving OD 50 at the antigen concentration of *[Ag]*.

2.6. *Production and purification of recombinant fragments of tetanus toxin*

For toxin fragment mapping of c-1F2C2, various fragments of tetanus toxin, including fragment B and fragment C as well as different subdomains of fragment C, including HCC and HCN were expressed in *E. coli* (Novagen, Germany). Here, the fragments were amplified from previously extracted genomic DNA of *C. tetani* (Yousefi et al., 2013) by PCR. The PCR reactions were performed in 25 μl volume containing 10× reaction buffer (2.5 μl), 50 mM $MgCl_2$ (1.2 μl), 10 mM dNTP (1 μl), Pfu DNA polymerase (0.2 μl), 10 pM forward and reverse primers (1 μl), 1:5 diluted DNA (1 μl) and DNase, RNase free water (17.1 μl). All materials and enzymes were purchased from Vivantis company (Malaysia). Primer sequences are listed in Table 5. The protocol of PCR includes an initial denaturation step of 3 min at 95 °C followed by 40 cycles, including a denaturation step at 94 °C for 30 s, an annealing step at 55 °C for 30 s and an extension step at 72 °C for 2 min and a final extension step at 72 °C for 10 min. The amplified fragments were cloned either into pET-28 b (+) vector (Invitrogen) (fragment C, HCN and HCC) or pET-32a (+) vector (Invitrogen) (fragment B) by restriction enzymes, including BamHI and HindIII (Thermo Fisher Scientific) and BL21 strain of *E. coli* was transformed with the construct. Afterwards, the transformed bacteria were selected by colony PCR.

Transformed bacteria were induced to express the recombinant fragments using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sinaclon). The optimal conditions yielding the maximal protein expression were determined at different temperatures (37 °C or 25 °C) and incubation times (3, 6 and 24 h) for each fragment. Thereafter, the large scale cultures of the bacteria were performed and the bacterial lysates were prepared using two buffers, buffer A and buffer B (Table 6). Expression of the recombinant proteins was evaluated by SDS-PAGE in the lysates obtained with buffers A and B. Next, the recombinant

Table 5
Sequences of the primers used for amplification of tetanus toxin fragments.

	Type of primer	Sequence (5′-3′)	Amplicon size
Fragment B	Forward	GGGTGTGGATCCTCATTAACAGATTTAGGAGGAG	1354 bp
	Reverse	GGGTGTAAGCTTAGAATAAGAAAATGGAATTGGTGTTG	
Fragment C	Forward	GGGTGTGGATCCGAAAAATCTGGATTGTTGGG	1221 bp
	Reverse	GGTGTGAAGCTTATCATTTGTCCATCCTTC	
HCN	Forward	GGATCCTAAAAATCTGGATTGTTGGGTTG	732 bp
	Reverse	AAGCTTATAACTTGTGTATAATTTTTCAATCTC	
HCC	Forward	GGATCCTTTATCTATAACCTTTTTAAGAGACTTC-3	621 bp
	Reverse	AAGCTTATCATTTGTCCATCCTTCATCTG-3	

HCN: N-terminal portion of fragment C, HCC: C-terminal portion of fragment C.

Table 6
Buffers and their components used for bacterial lysate preparations and recombinant protein purifications by Ni-NTA column.

Buffer	Imidazole (mM)	NaH_2PO_4 (mM)	NaCl (mM)	Tris base (mM)	Urea (M)
A	30	100	50	10	8
B	30	100	50	10	–
C	80	100	50	–	–
D	500	100	50	–	–
E	1000	100	50	–	–

Ni-NTA: Nickel nitrilotriacetic acid.

proteins were purified using nickel nitrilotriacetic acid (Ni-NTA) resin (Invitrogen). For purification of each recombinant protein by Ni-NTA, buffers with gradually increasing concentrations of imidazole from 30 mM to 1000 mM were used. The components of the buffers used for bacterial lysis and recombinant protein purification by Ni-NTA are presented in Table 6.

2.7. *Tetanus toxin fragment mapping of c-1F2C2*

Toxin fragment mapping of the MAb with the purified fragments was evaluated by ELISA and Western blot assays. The protocol used in the indirect ELISA was as follows: 1) Purified recombinant toxin fragments were coated on an ELISA plate (10 μg/ml) 2). Serially diluted concentrations of the antibody starting from 80 or 40 μg/ml were utilized in the experiment (the starting concentration of the antibody for fragment C was 10 μg/ml) 3) HRP-conjugated sheep anti-human or rabbit anti-mouse antibody at appropriate dilution (1:2000) were added to the primary antibody 4) TMB substrate solution was added to the wells, followed by addition of stop solution and OD measurement at 450 nm.

Western blot protocol used was as follows: 1) Purified recombinant fragments were loaded into the wells of a 12% SDS-PAGE gel (5 μg) 2) Transfer of the proteins onto the nitrocellulose membrane was carried out followed by blocking 3) MAbs and PAbs (2 μg/ml) were added to the membrane 4) HRP-conjugated sheep anti-human or rabbit anti-mouse antibody at appropriate dilution (1:1000) were added to the membrane 4) the bands were visualized by addition of ECL substrate and recorded on an autoradiography film.

2.8. In vivo *toxin neutralizing activity and curative effect of c-1F2C2*

In vivo tetanus toxin neutralizing activity of c-1F2C2 was examined as previously described (Ghotloo et al., 2019a). Briefly, 0.125 μg of each MAb was mixed with 10 minimal lethal dose (MLD) of the toxin (20000 pg) and incubated for 2 h at 37 °C. Thereafter, the mixture was intraperitoneally injected into female BALB/c mice (4–6 weeks old, 4 mice in each group) (Pasteur Institute of Iran) and the animals were monitored for the signs of paralysis followed by death for 30 days. Animals that survived until day 30 were considered fully protected against tetanus toxin challenge.

The second phase of *in vivo* neutralization experiments was performed to compare the potency of c-1F2C2 with that of the human anti-toxin PAb (Tetagam P). In this regard, various amounts of c-1F2C2 or the human anti-toxin PAb were mixed with various doses of the toxin, including 10 MLD and 2.5 MLD and intraperitoneally injected into mice (4 mice/group). The potency of c-1F2C2 was estimated by comparing the minimal amount of the MAb required for full protection of the animals against tetanus toxin with that of the human anti-toxin PAb.

Evaluation of the curative effect of c-1F2C2 was performed in a model in which 2.5 MLD of the toxin (5000 pg) was intraperitoneally administered to mice followed by intraperitoneal injection of various doses of the MAb or human anti-toxin PAb (Tetagam P, CSL Behring, USA) (3 mice/group). Animals were closely monitored for the signs of paralysis or death for 30 days. Decreasing the toxin dose from 10 to 2.5 MLD prolonged the disease course, providing sufficient time to evaluate the curative effect of the MAb when initial symptoms of the disease started.

Log rank test was used to analyze statistical differences between the groups.

In all experiments, animals were housed, handled, and treated according to the ethical standards of the Ethics Committee of Tehran University of Medical Sciences according to the principles of Helsinki Declaration.

2.9. *Inhibition of toxin binding to its receptors by c-1F2C2*

The ability of c-1F2C2 in inhibition of toxin binding to GT1b ganglioside receptors was evaluated by an indirect ELISA, as previously described (Yousefi et al., 2014a) and also by flow cytometry.

GT1b gangliosides (Sigma, St Louis, USA) (10 μg/ml prepared in ethanol) were coated on the wells of an ELISA plate and blocking was performed by 1% bovine serum albumin (Merck) in PBST. MAbs or PAbs at final concentrations of 250, 125, 62, 31, 15 and 7.5 μg/ml were preincubated with the toxin at final concentration of 20 μg/ml at 37 °C for 2 h, and then added to the wells. Thereafter, appropriate dilution (1: 1000) of HRP-conjugated human anti-toxin (PAb) (Sina biotech) wasadded to the wells. All incubations were performed at room temperature (except for the mixture of antibody and toxin which was incubated at 37 °C) for 2 h. Three times washings with PBST were carried out following each step. Finally, TMB substrate solution was added to the wells, followed by addition of stop solution and ODs of the samples were measured at 450 nm.

In addition, the ability of c-1F2C2 in binding to the pre-bound toxin on differentiated PC12 cells, which expressed tetanus toxin receptors (Herreros et al., 2000; Watanabe et al., 2018), was assessed by flow cytometry assay. PC12 cells (National Cell Bank of Iran, Pasteur Institute of Iran) were cultured in RPMI 1640 (Gibco, USA) medium supplemented with 10% heat–inactivated horse serum (Razi Vaccine and Serum Research Institute), 5% heat–inactivated fetal bovine serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). For the cells differentiation, 6×10^5 PC12 cells were seeded on T25 flasks pre-treated with Polyethyleneimine (PEI) solution (25 μg/ml) (Merck) for 1 h at 37 °C (Vancha et al., 2004) and exposed to nerve growth factor-β (NGF-β) (Sigma) at a final concentration of 400 ng/ml.

Exchange of the medium containing NGF-β was carried out every 2 days. At day 7 of the differentiation, the cells were detached from the flask using sodium citrate solution (0.01 M Sodium citrate, 0.3 M Potassium chloride). 100 μl solution of tetanus toxin at final concentration of 15 μg/ml was added to a total of 25 × 10^4 differentiated cell. Afterwards, a solution of the antibody at final concentration of 10 μg/ml (100 μl) was added to the cells. The final step included addition of fluorescein isothiocyanate (FITC)-conjugated anti-human antibody (Dako) to the cells. The incubation temperature and time in all steps were 4 °C and 1 h, respectively. The washing step with PBS was carried out following each step. The cells obtained from the final step were suspended in PBS and analyzed using a flow cytometer (Partec, Germany).

3. Results

3.1. Molecular cloning of c-1F2C2

To prepare the gene construct for the chimeric antibody, pBudCE4.1 vector containing c-1F2C2, VH and VL regions of the mouse MAb were amplified with degenerate primers that amplified all mouse VH and VL family genes. The amplified fragments were sequenced to design the specific primers for amplification of VH and VL regions that started from the leader sequences to the end of J regions. Afterwards, VH and VL regions of the mouse MAb were amplified by the designed primers (Fig. 1a).

To construct the mouse-human chimeric heavy and light chains, the human Cγ1 and Cκ were amplified (Fig. 1b) and joined to the mouse VH and VL regions, respectively, by SOEing PCR (Fig. 1c). Finally, the chimeric VH-Cγ1 and VL-Cκ segments were cloned into the pBudCE4.1 vector and the bacteria containing the construct were selected by colony PCR (Fig. 1d).

Cκ: Constant region of kappa light chainCγ1: Constant region of gamma 1 heavy chain, SOEing PCR: Splicing overlap extension polymerase chain reaction, VH: Variable region of mouse heavy chain, VL: Variable region of mouse light chain.

3.2. Expression and purification of c-1F2C2

The expression vector which contained c-1F2C2 construct was transfected into CHO–K1 cell line by Lipofectamin transfection reagent and production of chimeric antibody in the culture supernatant was assessed by ELISA. Our results showed that c-1F2C2 specifically recognized tetanus toxin (Supplementary Fig. 1a–b).

After expression of c-1F2C2 in CHO–K1 cells, the supernatant of the cells was collected and antibody purification was performed by SPG column. SDS-PAGE of the purified product under non-reducing conditions showed a major band with an approximate size of 150 KDa corresponding to an intact IgG molecule (Supplementary Fig. 1c) and 2 bands with approximate sizes of 50 KDa and 25 KDa corresponding to the heavy and light chains of IgG after antibody reduction (Supplementary Fig. 1d).

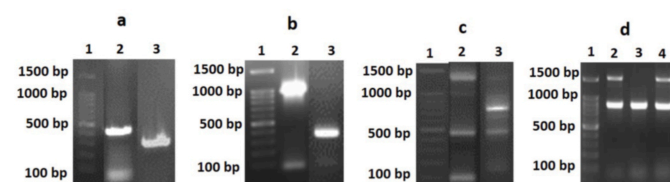

Fig. 1. Construction of the pBudCE4.1 vector containing c-1F2C2. a) Amplification of VH (lane 2) and VL (lane 3) regions of the mouse MAb; lane 1: size marker (Sinaclone). b) Amplification of Cγ1 (lane 2) and Cκ (lane 3) regions of the human IgG1 antibody; lane 1: size marker. c) Generation of chimeric VH-Cγ1 (1424bp) (lane 2) and chimeric VL-Cκ (718 bp) (lane 3) by SOEing PCR; lane 1: size marker. d) Selection of the bacteria carrying the vector containing both chimeric VH-Cγ1 (the upper band at approximate size of 1500 bp) and chimeric VL-Cκ (the lower band at approximate size of 1000 bp) by colony PCR; lane 1: size marker, lane 2, 3 and 4 are representative of bacterial clones.

3.3. Tetanus toxin binding and affinity constant determination of c-1F2C2

The specificity of c-1F2C2 to tetanus toxin was assessed in an indirect ELISA and Western blot. As shown in Fig. 2a, c-1F2C2 similar to its parental murine MAb recognized tetanus toxin. To confirm specific binding of the MAb to the toxin, Western blot was performed under non-reducing conditions. The results showed that the chimeric MAb similar to its murine counterpart, reacted with a band with approximate size of 150 KDa (the approximate size of a tetanus toxin molecule) and other bands at 70 KDa and 85 KDa (Fig. 2b), which were likely generated during toxin preparation procedure (Fig. 2c). Results of the reactivities of c-1F2C2 and 1F2C2 to toxin under reducing conditions showed a band with approximate size of 100 KDa corresponding to the approximate size of the toxin heavy chain (Fig. 2d). Both c-1F2C2 and 1F2C2 also recognized other bands at 70 and 75 KDa, which were probably generated during toxin preparation procedure (Fig. 2e).

In both ELISA and Western blot assays, human and mouse anti-tetanus PAbs served as positive controls.

ELISA: Enzyme-linked immunosorbent assay, PAbs: Polyclonal antibodies, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Affinity constant of c-1F2C2 was determined using an indirect ELISA. Based on 3 sigmoidal binding curves, the binding affinities for c-1F2C2 and 1F2C2 MAbs were very similar (8.7* 10^8 M^{-1} for c-1F2C2 and 9.5* 10^8 M^{-1} for 1F2C2) (supplementary Fig. 2a and 2b).

To assess insertion of human Cγ1 and Cκ into the chimeric MAb, an indirect ELISA was carried out. The results are presented in supplementary Fig. 3a and 3b.

3.4. Production of recombinant fragments of tetanus toxin and fragment mapping of c-1F2C2

To determine the target domains of the toxin recognized by c-1F2C2,

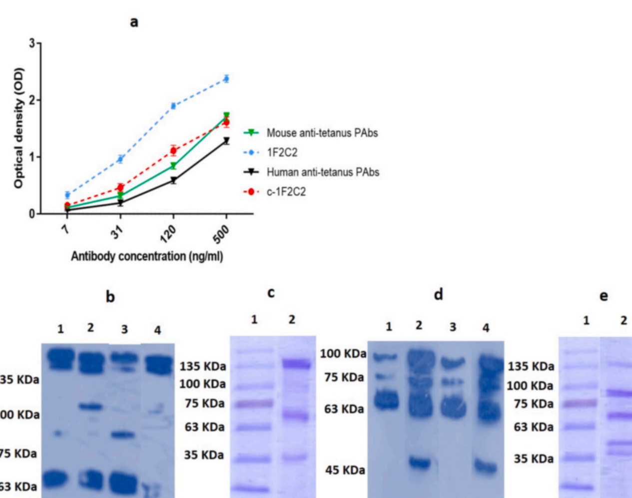

Fig. 2. Reactivity of c-1F2C2 with tetanus toxin. a) Reactivity of various concentrations of c-1F2C2 and its murine counterpart in an indirect ELISA. b) Reactivity of c-1F2C2 and its murine counterpart with non-reduced tetanus toxin by Western blot; lane 1: c-1F2C2, lane 2: human anti-tetanus PAb, lane 3: 1F2C2 and lane 4: mouse anti-tetanus PAb. c) SDS-PAGE pattern of the toxin under non-reducing conditions; lane 1: size marker and lane 2: tetanus toxins. d) Reactivity of c-1F2C2 and its murine counterpart with reduced tetanus toxin by Western blot; lane 1: c-1F2C2, lane 2: human anti-tetanus PAb, lane 3: 1F2C2 and lane 4: mouse anti-tetanus PAb. e) SDS-PAGE pattern of the toxin under reduced condition; lane 1: size marker and lane 2: tetanus toxin.

different toxin fragments, including fragment B and fragment C as well as two sub-domains of fragment C, including HCC and HCN were expressed in *E. coli*. The genes of various fragments of the toxin were amplified and cloned into pET vectors. The *E. coli* hosts containing different constructs were selected by colony PCR and expression of the proteins of interest were induced by IPTG. As shown in supplementary Fig. 4a–d, the fragments in expected sizes (a band in approximate size of 63 KDa for fragment B, 50 KDa for fragment C, 27 KDa for HCN and 20 KDa for HCC) were expressed and successfully purified by Ni-NTA resin.

After purification of all recombinant fragments of tetanus toxin, the reactivity pattern of c-1F2C2 with different fragments was evaluated and compared with that of 1F2C2 by ELISA and Western blot. c-1F2C2 and 1F2C2 were able to recognize fragment C in a comparable manner based on both ELISA (Fig. 3a) and Western blot (Fig. 3e). On the other hand, no binding to the other fragments of the toxin was observed for both c-1F2C2 and 1F2C2, while both mouse and human anti-toxin PAbs, which were used as positive controls, were able to recognize all fragments (Fig. 3 b-d and 3. e-h).

Western blot results of the reactivity of c-1F2C2 and its murine counterpart with non-reduced e) fragment C f) fragment B g) HCC sub-domain of fragment C h) HCN sub-domain of fragment C. In all figures: lane 1: c-1F2C2, lane 2: human anti-tetanus PAb, lane 3: 1F2C2 and lane 4: mouse anti-tetanus PAb.

ELISA: Enzyme-linked immunosorbent assay, HCC: Heavy chain C-terminal, HCN: Heavy chain N-terminal, PAb: Polyclonal antibody.

3.5. In vivo *tetanus toxin neutralizing activity and curative effects of c-1F2C2*

To evaluate tetanus toxin neutralizing activity of c-1F2C2, toxin neutralization assay was performed in female BALB/c mice. As shown in Figs. 4a and 0.125 μg dose of c-1F2C2 partially protected mice against 10 MLD of toxin, similar to MAb 1F2C2 at the same dose (p value = 0.09). Mice that received 10 MLD of the toxin without the MAb treatment died within 72 h of the toxin injection.

The results of *in vivo* toxin neutralization assay against 10 MLD of the toxin showed that the minimum amount of c-1F2C2 which could completely protect the mice against the toxin was 0.25 μg, whereas the minimum required dose of the human anti-toxin PAb which induced the same protection was 1 μg (Fig. 4b,supplementary fig. 5). Accordingly, a potency value of 0.77 IU/100 μg was obtained for c-1F2C2 versus 0.19 IU/100 μg for the commercial human anti-toxin PAb. In addition, 0.003

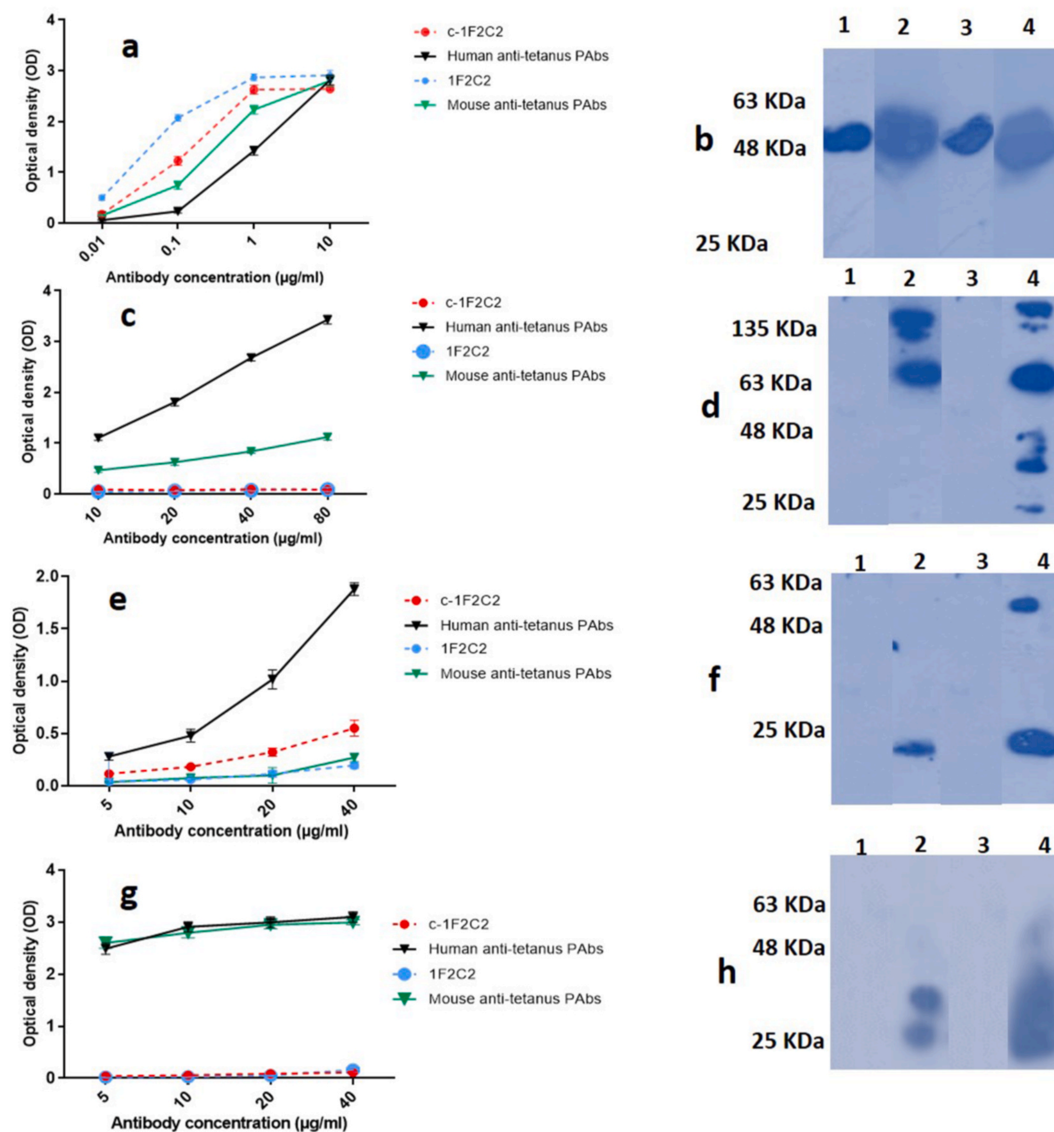

Fig. 3. Reactivity pattern of c-1F2C2 with different fragments of tetanus toxin. Reactivity of various concentrations of c-1F2C2 and its murine counterpart in an indirect ELISA with a) fragment C b) fragment B c) HCC sub-domain of fragment C d) HCN sub-domain of fragment C.

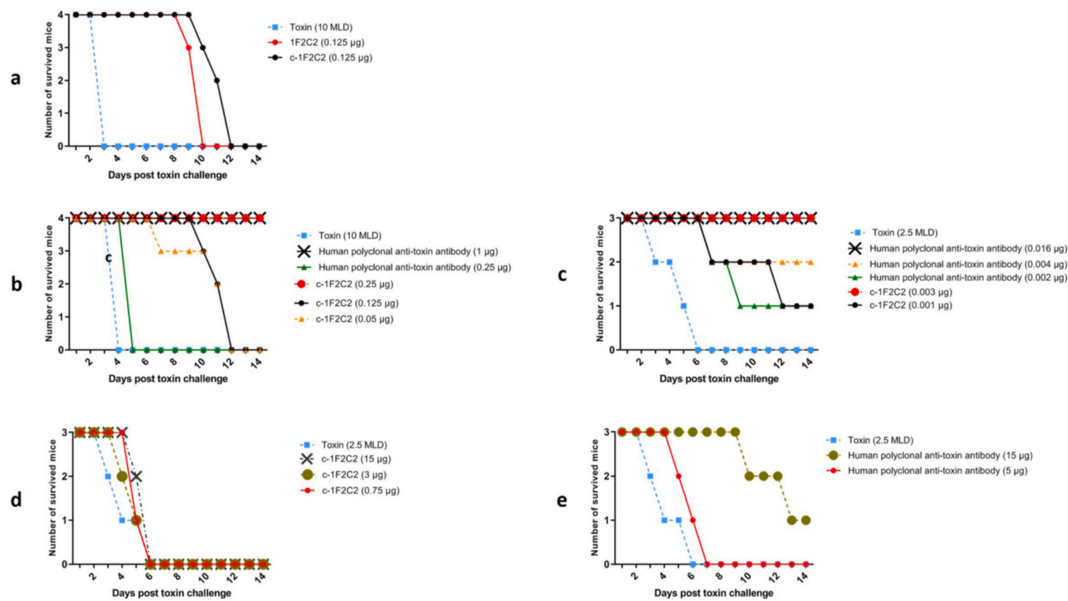

Fig. 4. In vivo tetanus toxin neutralizing activity and curative effect of c-1F2C2. BALB/c mice were intraperitoneally injected with a mixture of the antibody and tetanus toxin (toxin neutralizing effect of the antibody) or antibody preparation was intraperitoneally administered after toxin injection (curative effect of the antibody). a) Survival curves of the animals receiving a mixture containing the MAbs (c-1F2C2 or 1F2C2) and the toxin (10 MLD) (4 mice/group). b) Survival curves of the animals receiving a mixture containing tetanus toxin (10 MLD) and the antibodies (c-1F2C2 or human anti-tetanus PAbs) (4 mice/group). c) Survival curves of the animals receiving a mixture containing tetanus toxin (2.5 MLD) and the antibodies (c-1F2C2 or human anti-tetanus PAbs) (3 mice/group)d and e) Survival curves of the animals receiving various curative doses of c) c-1F2C2, d) Commercial human anti-tetanus PAb after 24 h of 2.5 MLD toxin administration (3 mice/group). MAb: Monoclonal antibody, MLD: Minimal lethal dose, PAbs: Polyclonal antibodies.

μg of the MAb could completely protect the animals against 2.5 MLD toxin, while the equivalent dose of the PAb was 0.016 μg (Fig. 4c).

To evaluate the curative effect of c-1F2C2, 2.5 MLD of the toxin was intraperitoneally injected to BALB/c mice, followed by administration of the Mab after 24 h. As shown in Fig. 4d, survival rates of the animals receiving various doses of c-1F2C2, including 0.75, 5 and 15 μg were not significantly different (p value > 0.05) and were similar to that of the control group receiving 2.5 MLD of the toxin alone. On the other hand, mice administered with 15 μg dose of human anti-toxin PAb induced significantly better protection compared with the group receiving 5 μg dose of the PAb (p value < 0.05) (Fig. 4e).

3.6. Inhibition of toxin binding to its receptors by c-1F2C2

Ganglioside GT1b is proposed as one of the main ganglioside receptors for toxin attachment and entrance into the cells (Montecucco, 1986). To determine whether *in vivo* toxin neutralizing activity of c-1F2C2 is mediated through its ability to inhibit binding of the toxin to its receptors, two assays were performed. In the first assay, the ability of c-1F2C2 to inhibit toxin binding to ganglioside GT1b was evaluated in

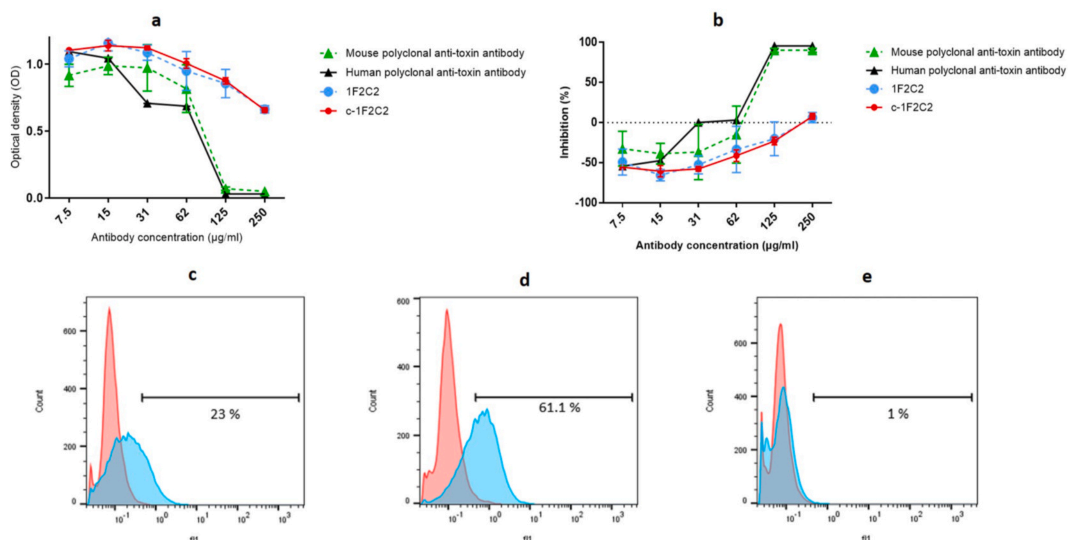

Fig. 5. Inhibition of tetanus toxin binding to its receptors by c-1F2C2 a-b) Evaluation of the ability of c-1F2C2 in inhibiting the toxin binding to GT1b ganglioside by an indirect ELISA in which GT1b ganglioside was coated on the wells of the ELISA plate a) The graph is plotted using the OD values obtained in the ELISA b) The graph is plotted according to the inhibition percent values of the toxin binding to GT1b gangliosides by c-1F2C2 c-e) Evaluation of the ability of the antibody in binding to the differentiated PC12 cells pre-incubated with the toxin by flow cytometry; c) c-1F2C2 d) human anti-tetanus toxin PAb e) an irrelevant isotype-matched MAb recognizing HER2.

an indirect ELISA. The results showed a minor inhibition (7%) of the toxin binding to the receptor at the highest tested concentration (250 μg/ml) for both c-1F2C2 and its murine counterpart (Fig. 5a–b).

In another assay, the ability of c-1F2C2 in binding to the differentiated PC12 cells pre-incubated with the toxin was evaluated by flow cytometry. As shown in Fig. 5c, c-1F2C2 bound to 23% of the cells, while human anti-toxin PAb bound to 61% of the cells (37% inhibition of the toxin binding) (Fig. 5d). An unrelated MAb recognizing human epidermal growth factor receptor 2 (HER2) protein, which served as a negative control, did not bind to PC12 cells (Fig. 5e).

PAb: Polyclonal antibody, ELISA: Enzyme-linked immunosorbent assay.

4. Discussion

In the present study, a previously generated murine anti-tetanus MAb (1F2C2) (Yousefi et al., 2014a) was manipulated to achieve a mouse-human chimeric form (c-1F2C2). This chimeric antibody showed higher potency in *vivo* toxin neutralization than that of the commercially available human anti-toxin PAb. "Tetanus toxin fragment mapping" analysis demonstrated that c-1F2C2 recognized fragment C of the toxin, while no reactivity with fragment B, HCN, and HCC sub-domains of fragment C was observed.

Currently, passive immunotherapy of tetanus is carried out by human anti-tetanus immunoglobulin which is a polyclonal preparation obtained from blood plasma of hyper-immunized donors (Ghotloo et al., 2019b). Utilization of this immunoglobulin preparation has several disadvantages and risks, including lot to lot heterogeneity, possibility of transmission of infectious agents from human plasma, adverse reactions to foreign proteins of plasma and the need for hyper-immunized donors who are required for plasma donation (Ghotloo et al., 2020) (Ghotloo et al., 2019b). Until now, no MAb preparation has been approved by the Food and Drug Administration (FDA) or European Medicines Agency (EMA) to replace the polyclonal preparations. Administration of MAbs with murine origin in human evokes HAMA response. Chimeric mouse-human MAbs may be considered as safer alternatives than mouse MAbs, since substitution of human constant regions of heavy and light chains for their murine counterparts reduces the immunogenicity and consequent HAMA response (Ghotloo et al., 2019b). These considerations prompted us to generate a mouse-human chimeric form of an anti-tetanus MAb. The structural and functional characteristics of the murine counterpart of this antibody were previously evaluated and the MAb showed strong *in vivo* toxin neutralizing activity.

Structural characterization of the chimeric antibody showed that human Cγ1 and Cκ domains were properly placed in c-1F2C2. In addition, c-1F2C2 showed similar toxin binding activity and affinity constant to its murine counterpart, indicating that the same VH and VL regions of murine MAb were inserted into the chimeric form. As mentioned, affinity constants of the MAb was determined using ELISA method. Although ELISA is practically more convenient than the other methods such as plasmon-surface resonance (PSR) and isothermal titration calorimetry (ITC) for the measurement of affinity constant, it is subjected to some limitations. While PSR and ITC methods measure signals in real time and require no labelling, ELISA is an end point detection method and signals are obtained in an indirect way. Moreover, multiple washing and incubation steps during ELISA may influence the interaction of low-affinity antibodies with their target antigens (Beeg et al., 2019; Heinrich et al., 2010).

The primary *in vivo* neutralization assays conducted with both forms of the MAbs demonstrated similar neutralizing effects for both antibodies, suggesting that the chimerization process of the mouse MAb was successful. In the next step, the potency of c-1F2C2 was estimated in comparison with the commercially available human anti-toxin PAb. Comparing the minimal amounts of c-1F2C2 and the commercial human anti-toxin PAb required for full protection of the animals against 10 MLD of tetanus toxin (0.25 μg for c-1F2C2 versus 1 μg for the commercial PAb) demonstrated a potency value of 7.7 IU/mg for c-1F2C2 and 1.9 IU/mg for the commercial human anti-tetanus PAb. Moreover, an estimated potency of 10 IU/mg for c-1F2C2 versus 1.9 IU/mg of the PAb was obtained when 2.5 MLD toxin was applied in neutralization assay. Although several mouse and human toxin neutralizing MAbs have so far been generated (Gustafsson et al., 1993; Kamei et al., 1990; Lang et al., 1993; Matsuda et al., 1992; Sheppard et al., 1984; Volk et al., 1984), a limited number were assessed for their potencies in comparison with polyclonal immunoglobulins and only one MAb has so far been reported to be able to individually confer a higher protection level against tetanus toxin than the polyclonal immunoglobulins (Luo et al., 2012). The higher toxin neutralization potency of c-1F2C2 may propose it as a suitable alternative to the current commercial human anti-tetanus PAb. Utilization of a single MAb as compared with a combination of MAbs reduces the production cost as well as the need for proper formulation.

Although the main purpose of using human anti-toxin PAb preparation is prophylaxis against tetanus, it has also been recommended as a therapeutic modality for tetanus patients soon after disease onset, but with no definite guaranteed curative outcome (WHO, 2010). Contrary to its prophylactic effects and similar to a MAb reported by Matsuda and colleagues, c-1F2C2 did not show substantial curative effect. This might be explained by the monoclonal nature of c-1F2C2, its affinity as well as the function of the epitope recognized by the MAb. Further evidence supporting this notion comes from. the toxin fragment mapping data and the results of. inhibition of the toxin bindings to its receptors by c-1F2C2 which suggest that neutralizing activity of c-1F2C2 against tetanus toxin is partly mediated through its ability to prevent the toxin binding to its receptors on target cells. In other words, while the MAb could interfere with the toxin binding to its receptors on the target cells and consequently prevents disease establishment in the animals, it failed to protect against the disease previously established in the animals. The toxin in the absence of MAb binds to its receptors on neurons through its free binding sites and subsequently inhibits release of the inhibitory neurotransmitters from neurons, resulting in the manifestation of tetanus in animals. On the other hand, the PAb preparation demonstrated a mild curative effect improving mouse survival rates to 33%. The better curative effect of the PAb is probably due to its polyclonal nature. Targeting different epitopes and fragments of the toxin by PAb may induce conformational changes in the toxin leading to its partial detachment from the neurons receptors. An anti-tetanus toxin MAb has also been reported which was able to induce 60% recovery in treated mice after 24 h of tetanus toxin injection (Lukic et al., 2015).

The Western blot results of reactivity of c-1F2C2 and its murine counterpart to the toxin under reducing conditions indicated binding to the heavy chain; the MAb recognized fragment C, but not fragment B of the heavy chain. Since fragment C of the toxin is responsible for binding of the toxin to its receptors on the neuronal cells, the toxin neutralizing effect of c-1F2C2 might be mediated through its ability to inhibit binding of toxin to its receptors. A considerable number of generated neutralizing MAbs against tetanus toxin are directed against fragment C (Gustafsson et al., 1993; Matsuda et al., 1992; Sheppard et al., 1984; Volk et al., 1984; Yousefi et al., 2014a, 2014b). A summary of these MAbs have recently been reviewed by Ghotloo et al. (2019b). Interestingly, further analyses have revealed that none of the fragment C sub-domains, including HCC, which is known for its role in toxin binding to its receptors (Sinha et al., 2000), and HCN was recognized by c-1F2C2. These results may suggest that the epitope recognized by this MAb is a conformational epitope of fragment C, which is destroyed upon dissociation of these sub-domains. Similar MAbs with specificity for either the light or heavy chain within the intact toxin molecule, but with no binding activity to the isolated chains, have already been reported (Arunachalam et al., 1992; Sheppard et al., 1984).

A dual receptor model has been proposed to explain attachment and entrance of tetanus toxin into neuronal cells. Based on this model, tetanus toxin uses two types of receptors to bind to the target cells, the ganglioside receptors, especially GT1b and GD1b gangliosides and the

protein receptors, including a GPI-anchored surface protein Thy-1 and synaptic vesicle protein 2 (SV2) (Blum et al., 2012, 2014; Lang and Jahn, 2009; Yeh et al., 2010). It has also been shown that toxin neutralizing effects of some anti-fragment C MAbs are mediated through the ganglioside receptors (Lukic et al., 2015; Luo et al., 2012). We observed that c-1F2C2 and its murine counterpart did not significantly inhibit binding of the toxin to GT1b ganglioside, even at the highest concentration tested (250 μg/ml). Interestingly, at lower concentrations, the MAb enhanced toxin binding to GT1b ganglioside. Our results suggest that the *in vivo* toxin neutralizing effect of c-1F2C2 is not mediated through GT1b ganglioside receptors. The antibody-mediated enhancement of toxin binding to GT1b might be due to a conformational change in the structure of the toxin upon antibody binding, facilitating its binding to the GT1b ganglioside. On the other hand, the results obtained from the binding of c-1F2C2 to differentiated PC12 cells pre-bound to the toxin revealed that the MAb was able to bind to a low proportion of the toxin-treated cells compared to the PAb. This suggests that the MAb's binding sites within the toxin are occupied by the receptors located on PC12 cells and are not fully accessible to the MAb. The lower signal obtained for the c-MAb could be due to the fewer epitopes available on Fc domain of the chimeric antibody compared to the whole human PAb for interaction with FITC-conjugated anti-human antibody. Therefore, it seems that the *in vivo* toxin neutralizing effect of c-1F2C2 might partly be conducted through the protein receptors and/or ganglioside receptors other than GT1b.On the other hand, the complete *in vivo* toxin neutralizing effect of the MAb versus its partial *in vitro* toxin neutralizing effect might be explained through *in vivo* clearance of the toxin-MAb immune complexes by phagocytes (Diamant et al., 2015).

5. Conclusion

The chimeric MAb c-1F2C2 displayed both *in vitro* and *in vivo* tetanus toxin neutralization effects and might be considered for passive immunotherapy of tetanus.

Author contributions

Somayeh Ghotloo designed and performed the experiments. Dr. Fazel Shokri developed and supervised the project. Dr. Forough Golsaz-Shirazi, Dr. Mohammad Mehdi Amiri and Dr. Mahmood Jeddi-Tehrani co-supervised the project. Somayeh Ghotloo, Dr. Fazel Shokri, Dr. Forough Golsaz-Shirazi and Dr. Mohammad Mehdi Amiri contributed to the writing and revision of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2021.08.011.

Ethics approval

This work was approved by the Ethics Committee of Tehran University of Medical Sciences. The animal experiments were conducted according to the ethical standards of the Ethics Committee of Tehran University of Medical Sciences according to the principles of Helsinki Declaration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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