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REVIEW ARTICLE

Epitope Mapping of Tetanus Toxin by Monoclonal Antibodies: Implication for Immunotherapy and Vaccine Design

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Abstract

Tetanus as a life-threatening disease is characterized by muscle spasm. The disease is caused by the neurotoxin of *Clostridium tetani.* Active form of tetanus neurotoxin is composed of the light chain (fragment A) and the heavy chain. Fragment A is a zinc metalloprotease, which cleaves the neuronal soluble N-ethylmaleimide-sensitive attachment receptor (SNARE) protein, leading to the blockade of inhibitory neurotransmitter release and subsequent generalized muscular spasm. Two functional domains of the heavy chain are fragment C, which is required for neuronal cell binding of the toxin and subsequent endocytosis into the vesicles, and fragment B, which is important for fragment A translocation across the vesicular membrane into the neuronal cytosol. Currently, polyclonal immunoglobulins against tetanus neurotoxin obtained from human plasma of hyper-immunized donors are utilized for passive immunotherapy of tetanus; however, these preparations have many disadvantages including high lot-to-lot heterogeneity, possibility of transmitting microbial agents, and the adverse reactions to the other proteins in the plasma. Neutralizing anti-tetanus neurotoxin monoclonal antibodies (MAbs) lack these drawbacks and could be considered as a suitable alternative for passive immunotherapy of tetanus. In this review, we provide an overview of the literature discussing epitope mapping of the published neutralizing MAbs against tetanus toxin.

Keywords Epitope mapping · Tetanus toxin · Monoclonal antibody · Immunotherapy · Vaccine design

Introduction

Tetanus is a bacterial infection manifesting itself by muscle spasms. It is caused by spores of the bacterium *Clostridium tetani* which exist in soil and animal intestinal tracts and contaminate many surfaces. These spores germinate in the anaerobic conditions of contaminated wounds into metabolically active bacteria which produce tetanus neurotoxin (Thwaites et al. 2015). The toxin prevents release of inhibitory neurotransmitters from neurons resulting in generalized muscular spasms observed in tetanus. Passive protection against tetanus toxin is obtained with polyclonal immunoglobulin preparations derived from human plasma of hyper-immunized donors (Lang et al. 1993). Given the limitations of these preparations (Lang et al. 1993; Kamei et al. 1990), monoclonal antibodies (MAbs) with neutralizing capacity against tetanus might be considered as an alternative source for passive immunotherapy of tetanus (Kamei et al. 1990; Lang et al. 1993).

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Tetanus Toxin Structure

Tetanus neurotoxin, which is produced by *Clostridium tetani*, causes the life-threatening disease of tetanus. Tetanus neurotoxin is synthesized as a 150-kDa single polypeptide chain and is subsequently cleaved to generate an active form of the toxin composed of the light chain (fragment A, 50 kDa) and the heavy chain (HC, 100 kDa), which are linked by a single disulphide bond (Yousefi et al. 2014b). The heavy chain consists of two functional domains, including the C-terminal domain (fragment C), which is required for neuronal cell binding and subsequent endocytosis into vesicles, and the N-terminal domain (fragment B), which is important for translocation of

fragment A across the vesicular membrane into the neuronal cytosol (Scott et al. 2010). Fragment C contains two subdomains, including the C-terminal sub-domain of HC (HCC) and the N-terminal sub-domain of HC (HCN). HCC is responsible for binding of the toxin to target cells (Fig. 1) (Yousefi et al. 2014a). Although the exact molecular mechanism for tetanus toxin entrance to the neurons is unknown, however, a dual receptors mechanism has been proposed, which includes a gangliosides receptor especially from GT1b and GD1b molecules and a protein receptor (Lalli et al. 1999; Chen et al. 2009; Petrusic et al. 2012).

Fragment A as a zinc metalloprotease cleaves vesicle-associated membrane protein-2 (VAMP-2), which is a neuronl soluble N-ethylmaleimide-sensitive attachment receptor (SNARE) protein, and therefore prevents release of inhibitory neurotransmitters such as glycine and gamma-aminobutyric acid (GABA). Furthermore, it has been shown that tetanus toxin inhibits neurotransmitter release by activation of neuronal transglutaminase (Ashton et al. 1995). Inhibition of neurotransmitters release leads to a spastic paralysis observed in tetanus (Petrusic et al. 2012).

Passive Immunotherapy of Tetanus

Humoral immunity provides protection against tetanus. Neutralizing antibodies bind to the toxin through variable regions and interfere with the attachment of the toxin to its receptors on the target cells and subsequent internalization to the cells (Pincus et al. 2014).

Passive immunotherapy with polyclonal immunoglobulins obtained from human plasma of hyper-immunized donors has been efficiently used for the prevention and therapy of tetanus (Lang et al. 1993). However, these preparations have many disadvantages, including high cost, the need to immunize donors, lot-to-lot heterogeneity, possibility of transmitting microbial agents, and the adverse reactions to the other proteins in the plasma (Lang et al. 1993; Kamei et al. 1990). Monoclonal antibodies (MAbs) against tetanus toxin lack the major drawbacks mentioned above and therefore might be considered as an alternative for passive immunotherapy (Kamei et al. 1990; Lang et al. 1993) (Table 1). Monoclonal antibodies could be generated from the murine origin by hybridoma technique or modified to chimeric and humanized forms by recombinant DNA technology or produced as fully human MAbs. Murine MAbs are not therapeutically as desirable as chimeric and human MAbs, due to elicitation of the human antibody response against the immunogenic murine epitopes leading to their inactivation and rapid clearance from circulation. Chimeric antibodies contain murine variable regions fused to human constant regions; this reduces immunogenicity of the chimeric antibodies in human. Humanized antibodies are generated by grafting hypervariable regions of murine antibodies on framework regions of human antibodies, resulting in a molecule of approximately 95% human origin (Arunachalam et al. 1992). Tetanus toxin is a large molecule which consists of different domains. This theoretically means that a large number of epitopes in the structure of the toxin would be able to elicit humoral immune response. Accordingly, it has been experimentally shown that a wide range of MAbs against different epitopes of tetanus toxoid can be isolated from a single individual or an animal model (Volk et al. 1984; Lang et al. 1993). Volk et al. isolated at least 20 hybridomas producing MAbs against distinct epitopes located on tetanus toxoid (Volk et al. 1984). In spite of generation of a wide variety of anti-tetanus MAbs in a single subject, only a small fraction of them are able to protect against tetanus toxin. For example, Volk et al. produced 32 MAbs against tetanus toxin, fragment B-II_b (corresponding to fragment C) and fragment I_{bc} (corresponding to fragment AB which consists of both fragment A and B), while only 9 of them gave a protection against lethal challenge of the toxin in mice (Volk et al. 1984). Similarly, in another study, only 6 of 100 anti-

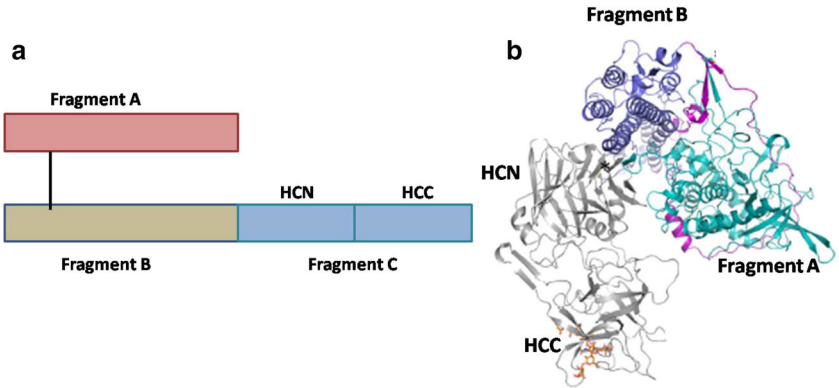

Fig. 1 Tetanus toxin structure. **a** Schematic representation: tetanus toxin contains two chains including the light chain (fragment A) and the heavy chain. The heavy chain consists of two functional domains, including fragment C, which is required for neuronal cell binding and fragment B, which is responsible for translocation of fragment A across the vesicular membrane into the cytosol. Fragment C is composed of two subdomains, including HCC and HCN. HCC contributes in the binding of the toxin to neurons. **b** Three-dimensional structure (adapted from Thwaites et al. 2015)

Table 1 Major features of reported tetanus toxin neutralizing monoclonal antibodies

MAb clone	MAb species	Immunizing antigen	Toxin dose used in mice	MAb dose used for toxin neutralization in mice	Epitope recognized by MAb	Isotype of MAb	Affinity of MAb	Reference number
7-T, 16-T	Mouse	Tetanus toxoid	L+/1000	0.32 μg	Fragment B-Ibc	IgG2a	NI	12
9-T, 26-T	Mouse	Tetanus toxoid	L+/1000	3.2 μg	Fragment B-Ibc	IgG1	NI	12
11-T, 17-T, 22-T	Mouse	Tetanus toxoid	L+/1000	3.2 μg	Fragment B-Ibc	IgG2a	NI	12
52-T	Mouse	Tetanus toxoid	L+/1000	3.2 μg	Fragment B	IgG2b	NI	12
56-T	Mouse	Tetanus toxoid	L+/1000	3.2 μg	Fragment B	IgG1	NI	12
19-B-IIb	Mouse	Fragment B-IIb (fragment C)	L+/1000	3.2 μg	Fragment B-IIb	IgG1	NI	12
TT03	Rat	Tetanus toxoid	L+/1000	NI	Fragment C	IgG1	NI	16
TT10	Rat	Tetanus toxoid	L+/1000	NI	Fragment C	IgG1	NI	16
TT06	Rat	Tetanus toxoid	L+/1000	NI	Fragment C	IgG1	NI	16
TT09	Rat	Tetanus toxoid	L+/1000	NI	Fragment C	IgG1	NI	16
G2	Human	Tetanus toxoid	20 MLD	0.089 μg	Fragment C	NI	11×10^{10} M^{-1}	17
G1	Human	Tetanus toxoid	20 MLD	0.89 μg	Fragment B	NI	8.3×10^{10} M^{-1}	17
G3	Human	Tetanus toxoid	20 MLD	2.8 μg	Fragment B	NI	2.9×10^{10} M^{-1}	17
G6	Human	Tetanus toxoid	20 MLD	0.028 μg	Fragment B	NI	10×10^{10} M^{-1}	17
G4	Human	Tetanus toxoid	20 MLD	2.8 μg	Fragment A	NI	14×10^{10} M^{-1}	17
F5/S/8E10	Human	Tetanus toxoid	500 LD50	16.8 ng	NI	IgG	NI	11
143	Human	Tetanus toxoid	L+/1000	NI	Fragment C	IgG1	NI	15
147	Human	Tetanus toxoid	L+/1000	NI	Fragment C	IgG1	NI	15
ST15	Human	Tetanus toxoid	450 MLD	NI	Fragment A and fragment C	IgG1	5.6×10^{9}	9
1F2C2	Mouse	Tetanus toxoid	10 MLD	10 μg	Fragment C	IgG2a	7.1×108	3
1F1E12	Mouse	Tetanus toxoid	10 MLD	10 μg	Fragment C	IgG2a	8.1×108	3
1F2C8	Mouse	Tetanus toxoid	10 MLD	10 μg	Fragment C	IgG2a	1.9×109	3
51	Mouse	Tetanus toxoid	2 LD50	10 μg	Light chain	IgG1	2.4×108	19

L+/1000 the smallest quantity of the toxin that when mixed with 0.001 IU of anti-toxin antibody causes the death of the test animals within 96 h, *MLD* minimal lethal dose of the toxin, *LD50* the smallest quantity of the toxin, that when administered by the specified route, causes the death of 50% of the test animals within a given period, *NI* not identified

tetanus MAbs showed a protective capacity against tetanus (Lang et al. 1993). We have recently generated a panel of 22 mouse hybridoma clones specific for tetanus toxin, but only 3 of the MAbs displayed toxin neutralizing activity in an animal model (Yousefi et al. 2014a; Yousefi et al. 2014b; Yousefi et al. 2016). Therefore, it seems necessary to determine which epitopes of tetanus toxin are able to elicit protective antibodies.

Protective Epitopes Located on Fragment C of Tetanus Toxin

Generally, protective antibodies against toxins are considered to have neutralization capacity. In this regard, antibody-mediated neutralization of the toxin is dependent on the ability of the antibody to bind to the toxin and inhibits the attachment of toxin to its cellular receptors and thereby preventing the toxin from cellular entrance and subsequent pathological effects. Therefore, we can assume that MAbs directed against epitopes located on fragment C, which harbors receptor binding domain of tetanus toxin, have the ability to neutralize the toxin. In this context, blocking the receptor binding site of the toxin by its absorption to GT1b, GD1b, and GM1 gangliosides results in loss of neutralizing capacity of anti-tetanus toxin MAbs in animal models (Petrusic et al. 2012). Recently, we established a MAb designated 1F3B3, against fragment A of the toxin, which was not able to inhibit the binding of tetanus toxin to GT1b gangliosides and failed to neutralize tetanus toxin in an animal model (Yousefi et al. 2014b). Results of another study showed that almost 42% of anti-fragment C MAbs, which were isolated from the spleen of the mice immunized with tetanus toxoid, were able to protect BALB/c mice from L+/1000 dose of the toxin (Volk et al. 1984), the smallest quantity of the toxin that when mixed with 0.001 IU of anti-toxin antibody causes the death of the test animals within 96 h (Council of Europe 2010). Interestingly, when fragment C instead of the whole toxin was used to immunize mice, approximately 13% of anti-fragment C MAbs

were able to protect the mice against a dose of L+/1000 of the toxin. These results suggest that three-dimensional structure of fragment C, which has been enzymatically digested, might be slightly different from that of the intact toxin. Therefore, some antibodies generated against epitopes within the isolated fragment C were not able to bind to the epitopes in the intact fragment C region within the whole toxin and consequently could not neutralize its toxic effect (Volk et al. 1984).

In another study, epitope mapping of two human anti-tetanus toxin MAbs, which could individually protect the mice from either paralysis or death against L+/1000 dose of the toxin within 4 days, showed that both MAbs recognized epitopes in fragment C and inhibited its binding to GT1b receptors. These MAbs recognized two distinct epitopes of fragment C, suggesting that at least two different epitopes of fragment C are involved in the tetanus toxin binding to GT1b receptors and blocking any of these two epitopes would confer protection against tetanus toxin. Furthermore, by comparison with the WHO international reference preparation of anti-tetanus toxin, the protective activity of these MAbs was estimated between 100 and 120 international units (IU) per milligram (mg) of antibody (Gustafsson et al. 1993). Results of another study conducted by Sheppard et al. were in line with the previous one, showing that there is at least two protective epitopes in fragment C. Four generated MAbs in this study recognized two distinct epitopes of fragment C, designatedTT03-TT10 and TT06-TT09, as appeared from the results of competition ELISA; these MAbs were able to protect the mice against lethal challenge of the toxin (Sheppard et al. 1984). MAb TT04, which recognized the third epitope of fragment C, could not protect the animals from the lethal effect of the toxin, explaining that not all epitopes located in fragment C can trigger production of neutralizing antibodies. Two other MAbs (TT05-TT08) moderately inhibited binding of labeled MAb TT03 and MAb TT10 to the toxin, suggesting that their targeted epitope is in close proximity to that of TT03-TT10; however, none of them reacted with either isolated heavy or light chain of the toxin. This may indicate that both MAbs are directed against the conformational epitopes presented on the intact molecule (Sheppard et al. 1984). MAb-G2 is another anti-tetanus MAb generated by Matsuda et al. and is one of the so far reported MAbs with the highest neutralizing activity. The MAb recognizes an epitope located on fragment C and can rescue the mice from tetanus with a minimum dose of 0.089 μg in an in vitro neutralization test in which 20 MLD of tetanus toxin was incubated with the antibody, and the mixture was intramuscularly injected to animals (Matsuda et al. 1992).

Anti-tetanus toxin MAbs are useful tools in exploring relationship between tetanus toxin structure and function. In order to further characterize GT1b binding site of fragment C, 13 MAbs, all recognizing epitopes located on fragment C, were generated by Shapiro et al. Eight MAbs which completely inhibited biotin-labeled recombinant fragment C binding to immobilized ganglioside GT1b, recognized overlapping epitopes defined as epitope 1. Sequencing of variable region genes demonstrated that the variable region of heavy chain (VH) and kappa light chain (Vk) of all these MAbs belonged to V_HQ52N and Vκ12/13 family, respectively. On the contrary, two other MAbs including 35F7 and 18.2.12.6, which had the same V_H and Vκ families (V_HJ558 family and Vκ9A family), did not compete with each other in binding to recombinant fragment C in a competition ELISA, suggesting that they recognized distinct epitopes of fragment C. Interestingly, they exerted opposite effect on the binding of fragment C to ganglioside GT1b. While MAb 35F7 completely inhibited binding of fragment C to ganglioside GT1b, MAb 18.2.12.6 enhanced its binding. This enhancement might be due to a conformational change in the structure of fragment C upon antibody binding, facilitating its binding to the gangliosides. In other words, MAbs utilizing the same V_H and V_L families in their variable region genes may not bind to the same epitope. The epitopes recognized by MAbs 35F7 and 18.2.12.6 were designated epitope 2 and 3, respectively. Three other MAbs generated in this study, including 72B9, 81H10, and 18.1.7 recognized overlapping epitopes based on the results of competition ELISA. In spite of recognizing overlapping epitopes, only two of three MAbs (81H10 and 18.1.7) which utilized V_H36-60 and Vκ19/28 gene families were able to inhibit binding of fragment C to GT1b gangliosides. Thus, the epitope recognized by these MAbs were designated epitope 4. In spite of the ability of MAbs 81H10 and 18.1.7 in abolishing the binding of fragment C to gangliosides either completely for 81H10 (complete abolished binding of fragment C at concentration of 2.5 μg/ml) or partially for 18.1.7 (complete abolished binding of fragment C at concentration of 20 μg/ml), MAb 72B9 did not block fragment C's binding to gangliosides. The difference in inhibiting the binding of fragment C to GT1b gangliosides between 18.1.7 and 81H10 may be partly explained by the fusion partner utilized to immortalize hybridoma 18.1.7. It was P3-X63Ag8 myeloma which expresses endogenous IgG1,κ antibody. Therefore, the antibodies generated from this hybridoma are a mixture of the endogenous antibody and specific antibody (18.1.7), and consequently, more concentrations of the MAb are required to achieve complete inhibition of fragment C binding. MAb72B9 used Vκ4/5 family, which was different from that of MAbs 81H10 and 18.1.7, and it recognized a distinct epitope of fragment C designated epitope 5. It is probable that this epitope was too close to targeted epitope of MAbs 81H10 and 18.1.7 which was distinguished by a competition ELISA. Altogether, these data suggest that at least three distinct epitopes of fragment C are involved in forming GT1b ganglioside binding site of the toxin; however, no in vivo experiment was performed to determine the neutralizing potency of these MAbs. In vivo experiments might help to identify whether

different epitopes of fragment C which participate in tetanus toxin binding to GT1b gangliosides receptors have the same pattern of receptor binding and toxin neutralization. These experimental data have shown that 3 sets of assays, including competition assay, functional assay, and determining V_H and V_L families, should be performed to define whether distinct or the same/overlapping epitopes are recognized by a set of the MAbs (Fitzsimmons et al. 2000).

Six out of 7 anti-fragment C antibodies generated in the study of Yousefi et al. inhibited tetanus toxin binding to GT1b gangliosides either completely (1F3E3, 1F2C2, 1F1E12, and 1F2C8) or partially (2C9B6 and 3B3D9). Results of the in vivo neutralization study showed that MAb 1F3E3, which displayed the highest inhibitory activity in GT1b binding assay, was not able to neutralize the toxin in mice. Three other fragment C specific MAbs including 1F2C2, 1F1E12, and 1F2C8 were completely able to neutralize the toxin, whereas 2C9B6 and 3B3D9 MAbs only resulted in a partial protection against tetanus toxin in the animals. These results suggest that anti-tetanus MAbs which inhibit attachment of the toxin to GT1b gangliosides are more effective in the toxin neutralization in animals. No protective ability of MAb 1F3E3 in the in vivo experiment may indicate involvement of the other receptors in the entrance of tetanus toxin to neuronal cells. Surprisingly, epitope mapping of these MAbs against HCC subdomain of fragment C revealed that only one of 6 MAbs (1F3E3) showed positive reactivity to HCC subdomain. As HCC subdomain contains key amino acids responsible for the binding of tetanus toxin to GT1b gangliosides, the inhibitory activity of 5 other MAbs to the gangliosides can be explained through steric hindrance of FC region on the GT1b ganglioside binding site of the toxin or conformational changes in GT1b binding domain of the toxin resulting in the prevention of its binding to the gangliosides (Yousefi et al. 2014b; Yousefi et al. 2014a; Yousefi et al. 2016).

Analysis of the MAbs against fragment C proposed that GD1b gangliosides might be as essential as GT1b gangliosides in mediating entrance of tetanus toxin into neurons and their blocking led to protection of mice against tetanus toxin. MAb 51 with ability to protect against 2-fold 50% lethal dose of tetanus toxin (2LD50), almost completely inhibited tetanus toxin binding to GD1b gangliosides (Lukic et al. 2015). Furthermore, 10 μg of MAb 51 resulted in complete recovery of 100% of mice from the pathology of tetanus, when applied 2 and 6 h after tetanus toxin intoxication and 60% after 24 h of tetanus intoxication. Interestingly, three other MAbs in this study including MAb 33, 39, and 71, which exerted partial protection against tetanus with the survival rates of 80%, 80%, and 60%, respectively, partially inhibited binding of tetanus toxin to GD1b gangliosides (almost 50–60%). Accordingly, they were able to partially protect against tetanus toxin challenge, following 2 and 6 h of intoxication (survival rates between 40 and 80%). Conversely, another MAb designated MAb 41, which completely inhibited toxin binding to GD1b receptors, did not protect the mice from 2LD50 of tetanus toxin, suggesting that GD1b gangliosides are not the sole receptors mediating entrance of tetanus toxin to neuronal cells (Lukic et al. 2015).

Another line of evidence supporting the idea that at least some of the protective epitopes of tetanus toxin are located in fragment C comes from the studies in which recombinant or purified native fragment C has been utilized as a vaccine candidate. In this regard, functionality of a subunit vaccine composed of recombinant fragment C was evaluated in a ganglioside binding assay. Results of this functional assay showed that binding affinity of recombinant fragment C to GT1b gangliosides was as strong as that of tetanus toxin. Thereafter, efficacy of two doses of fragment C-vaccine was compared to the equivalent doses of toxoid-vaccine. The results showed that while vaccination with the equivalent doses of toxoid resulted in a partial protection against a subcutaneous challenge of 100,000-fold of LD50 in the mice, fragment C-vaccine provided 100% survival rate in all vaccinated animals. Accordingly, more elevated levels of anti-fragment C and anti-toxoid antibodies titer were observed in fragment C- vaccinated mice. Furthermore, the titer of neutralizing antibodies against fragment C in vaccinated mice was higher than those in the mice receiving toxoid-vaccine (≥ 0.25 IU/ml for fragment C- vaccinated mice compared with ≤ 0.125 IU/ml for toxoid-vaccinated mice). Results of a detailed study on evaluating efficacy of the recombinant fragment C vaccine using alhydrogel adjuvant revealed that a single injection of 1 μg of fragment C-vaccine was able to provide complete protection against 1000 LD50 of the toxin, while two injections of as few as 0.04 μg of the vaccine resulted in almost complete protection of the mice against 10,000 and 100,000 LD50 of the toxin. Furthermore, the serum anti-tetanus antibody of fragment C-vaccinated mice inhibited binding of fragment C and toxin to GT1b gangliosides even more effectively than that of toxoid-vaccinated mice, indicating that the neutralizing capacity of fragment C-vaccine was associated to its potential in eliciting antibodies with ability to block binding of fragment C to its gangliosides receptors (Yu et al. 2011; Fairweather et al. 1987).

Another study has shown that a vaccine composed of fragment C domain alone would be as efficient as the vaccine consisted of fragment C and other domains of the toxin in providing protection against tetanus toxin. Fairweather et al. evaluated immune response and protective capacity of two tetanus toxin fragments including purified fragment C and a 63 kDa recombinant protein consisting of all 451 residues of fragment C as well as 121 residues of fragment B (residues 743 to 1314). Their results showed that immunization of mice with an equal level of purified fragment C or 63 kDa

recombinant protein elicited a comparable level of antibody and protection indicating that fragment C domain alone would be able to provide protection against tetanus toxin (Fairweather et al. 1987).

It has been demonstrated that some derivatives of fragment C of tetanus toxin (TetC), including TetC1-451, TetC1-271, TetC1-180, and TetC80-451 could induce protective immunity in the mice (Figueiredo et al. 1995). This suggests that a subunit vaccine spanning TetC80-180 of tetanus toxin might provide protection against tetanus, although its protective ability against tetanus in human remained to be evaluated. To investigate the mechanism by which TetC1-451, TetC1-271, TetC1-180, and TetC80-451 provide protection, the binding properties of these recombinant fragments to primary neurons were evaluated. While TetC1-451 and TetC80-451 bound to the primary neurons, TetC1-271 and TetC1-180 were not able to bind to neurons, suggesting that protective effect of antibodies against TetC1-451 and TetC80-451 was probably mediated through inhibition of the binding of toxin to its receptors (Figueiredo et al. 1995). It seems that TetC80-271 contains a critical region which is necessary for binding of fragment C to its receptor. The other recombinant fragments including TetC1-92 and TetC391-451 were inactive in this binding assay. Recombinant fragment TetC1-405, which lacks 46 carboxy-terminal residues of fragment C, showed poor ability in providing protection against tetanus. This is probably related to its poor solubility especially that immunization with this derivative induced low level of specific antibody (Figueiredo et al. 1995).

Protective Anti-Tetanus MAbs Against Regions Other Than Fragment C

It has been shown that anti-tetanus MAbs directed against regions other than fragment C of the toxin are also able to neutralize tetanus toxin. Although the exact mechanisms of their neutralizing capacity is not clear, it is possible that one or more of the following steps is prevented by these MAbs (Volk et al. 1984): (1) transport of the toxin across the cell membrane; (2) retrograde transport of the toxin along the nerve fibers to its targets; (3) blocking the action of the toxin on its target. Additionally, it is possible that binding of these MAbs to the toxin lead to a conformational change on the receptor binding domain of the toxin and subsequently prevent binding of tetanus toxin to its receptor (Volk et al. 1984). Furthermore, it has been suggested that the other regions of the toxin except fragment C might contribute to its binding to the neuronal membrane and consequently their blocking led to the inhibition of tetanus toxin binding to its receptors (Helting et al. 1977; Pellizzari et al. 1999; Blum et al. 2014). One of the evidences comes from a study in which a significantly higher concentration of fragment C than the whole toxin was required to achieve the same effects of the toxin in the binding to the gangliosides, neural membranes, and retrograde axonal transport (Helting et al. 1977). Accordingly, fragment C did not inhibit retrograde axonal transport of whole toxin (Pellizzari et al. 1999).

Results of a study showed that a large proportion of neutralizing MAbs generated against tetanus toxoid (almost 67% of all generated MAbs) were directed against distinct epitopes of fragment B. These MAbs were able to protect the mice from a L+/1000 dose of the toxin. Interestingly, when recombinant Ibc fragment which contains fragment A and B of the toxin was used to immunize mice against tetanus, none of the isolated MAbs could protect the mice from the L+/1000 dose of the toxin, suggesting that three-dimensional structure of isolated fragment Ibc may slightly differ from that of the intact molecule. Therefore, generated MAbs against epitopes of fragment Ibc would not be able to bind to antigenic determinants of this fragment in the intact molecule and consequently neutralize its toxic effect (Volk et al. 1984). In another study, 50% of generated MAbs with neutralizing capacity against tetanus toxin were directed against fragment B. These included MAbs G1, G3, and G6 which were able to protect the mice from 20MLD of toxin in doses as minimal as 0.89, 2.8 and 0.028 μg, respectively (neutralizing potency of these MAbs was 1, 0.3, and 30 IU/70 μg, respectively). In addition, a curative effect was observed following an IV injection of 0.03 IU of MAbs G2 and G6 within 6-10 h of intoxication of mice with 4MLD of toxin (Matsuda et al. 1992).

In addition to neutralizing MAbs directed against fragment B, protective MAbs against fragment A of the toxin were also identified. Using a neutralization inhibition test, Hardgree et al. estimated that about 25% of total neutralizing antibodies in human tetanus immunoglobulin (TIG) are directed against fragment A in light chain of the toxin (Lin et al. 1985). Lang et al. demonstrated that neutralizing capacity of TIG was primarily conferred by anti-fragment A antibodies, since protective capacity of TIG was completely abolished by addition of 2 μg fragment A; however, absorption of TIG with eight times higher concentration of fragment C than that of fragment A had no significant effect on its potency (Lang et al. 1993). They also demonstrated that a majority of anti-tetanus MAbs produced by EBV transformation of human B cells were directed against epitopes located on fragment A in light chain of the toxin (> 90%) and 16% of clones exhibited partial neutralizing activity resulted in a delay in the death of the mice intoxicated with 45MLD of toxin (neutralizing activity of antibodies was in the range of 2.4 to > 5.5 IU/100 μg) (Lang et al. 1993). Furthermore, one of these MAbs designated ST15 was able to provide partial protection against higher levels of the toxin (450MLD). Since all the MAbs belonged to the same sub-class (IgG1) and no correlation between their affinities and neutralizing activities was observed, the differences in neutralizing activity between these MAbs are probably related to the targeted epitopes localized on fragment A. This suggests

that some epitopes of fragment A are much more crucial than the others in elicitation of protective antibodies against tetanus (Lang et al. 1993). Interestingly, the only MAb which was able to completely give protection against tetanus recognized an epitope located in both fragments C and A, suggesting that there might be a shared epitope between fragments C and A of the toxin (Lang et al. 1993). The estimated neutralizing activity of ST15 MAb was ranged between > 5.5 and 13.2 IU/100 μg IgG for 45MLD and 450MLD of toxin, respectively (Lang et al. 1993). The concept of presence of a shared epitope between fragment C in heavy chain and fragment A in light chain of tetanus toxin was proposed for the first time by Scrivner et al. (Scrivner et al. 1987).

The other reported protective MAb against fragment A, MAb-G4, was able to protect the mice against 20 MLD of tetanus toxin at a minimum dose of 2.8 μg (equivalent to a potency of 0.3 IU/70 μg of IgG). An IV dose of 0.03 IU of MAb-G4 suppressed the progression of tetanus symptoms following 10 h of mice intoxication with 4MLD of the toxin. The MAb treatment within 18–24 h of intoxication, only delayed the symptoms' progression and finally death of the mice was occurred (Matsuda et al. 1992). It seems that protective epitopes of fragment A are distinct from the epitopes retaining its enzymatic activity. Vaccination with two doses of a recombinant form of fragment A incorporating a Glu-234 to Ala mutation in active site of the enzyme, which leads to abolishment of enzymatic activity of fragment A, resulted in complete protection of the mice against LD50 of tetanus toxin, the same as the recombinant unmutated form of fragment A (Figueiredo et al. 1995).

In addition to protective MAbs recognizing epitopes located on fragment C, B, or A, there are protective MAbs which reacted neither with the isolated epitopes of heavy chain nor with those of light chain. This suggests that targeted epitopes of these MAbs are conformational epitopes which are destroyed upon isolation of heavy and light chains of the toxin (Arunachalam et al. 1992).

It is notable that the epitope mapping of a neutralizing MAb may not mechanistically explain neutralizing capacities of the MAb (Fig. 1), since its neutralizing capacity might result from the following: (1) the steric hindrance of the Fc region of MAbs on the receptor binding site of the toxin upon binding of MAbs to the targeted epitope (Fig. 2c and d) or (2) conformational change of the receptor binding site of the toxin after binding of MAb to the toxin, which might lead to abolished or reduced binding of toxin to its receptors (Fig. 2e). The direct evidence for this phenomenon comes from a study in which 4 of 5 single-chain variable fragments (scFvs) clones directed against HCN sub-domain of fragment C were able to reduce binding of fragment C to gangliosides; therefore, in addition to scFvs against HCC, which is responsible for the binding of fragment C to its receptor, scFvs against HCN sub-domain could be protective (Yousefi et al. 2014b). Epitope mapping of a MAb, MAb 51, with different fragments of tetanus toxin in a western blot analysis showed that this MAb can recognize an epitope in fragment A of the toxin. This finding is in sharp contrast with the ability of MAb 51 in complete inhibition of tetanus toxin binding to GD1b gangliosides, since the binding properties of tetanus toxin are attributed to fragment C, which is located on the heavy chain. These findings can be explained through the steric hindrance of intact antibody on GD1b ganglioside-binding site of the toxin or alternatively a conformational change on GD1b ganglioside-binding site of the toxin upon its binding to the toxin (Lukic et al. 2015).

Results of our study showed that neither Fab nor (Fab′)2 fragments of 3 MAbs including 1F2C2, 1F3E3, and 2C9B6, either individually or in combination, were able to protect mice against 10MLD of tetanus toxin even at 10 μg. However, 0.5 μg of 1F2C2, 2.5 μg of 2C9B6, and 0.25 μg of each antibody in the combination of 1F2C2 and 1F3E3 was able to completely protect the animals. The fact that Fab and (Fab′)2 fragments of polyclonal anti-tetanus immunoglobulin were able to fully protect the mice against 10MLD of toxin, similar to their intact antibody suggests that loss of protective activity of our MAbs' fragments is not directly attributed to their Fc region. Steric hindrance of Fc region and/or conformational change of the receptor binding site of the toxin by Fc regions may explain toxin neutralization by the MAbs (unpublished data).

Synergistic Protective Effects of a Combination of Anti-Tetanus Monoclonal Antibodies

Polyclonal pool of human anti-tetanus immunoglobulin contains antibodies directed against different epitopes of tetanus toxin, but only a small fraction of the antibodies are capable of neutralizing tetanus toxin. Replacement of human polyclonal anti-tetanus immunoglobulin with anti-tetanus MAbs is feasible only if one or a limited number of anti-tetanus MAbs could confer protection at a level comparable to the human polyclonal anti-tetanus immunoglobulin. So far, a few MAbs have been reported which were individually able to confer a protective activity similar or higher than that of human polyclonal anti-tetanus immunoglobulin (Luo et al. 2012). It has been shown that a combination of two or more MAbs may synergistically augment the neutralizing activity against tetanus toxin compared to a single MAb. The synergism might occur between two or more non-neutralizing MAbs which are individually unable to protect against tetanus toxin. Indeed, utilizing a combination of these non-neutralizing MAbs was able to provide protection against tetanus toxin challenge. Ziegler-heitbrock et al. observed that while none of the generated antibodies against fragments B and C located on heavy and light chains of the toxin, respectively, was individually able to

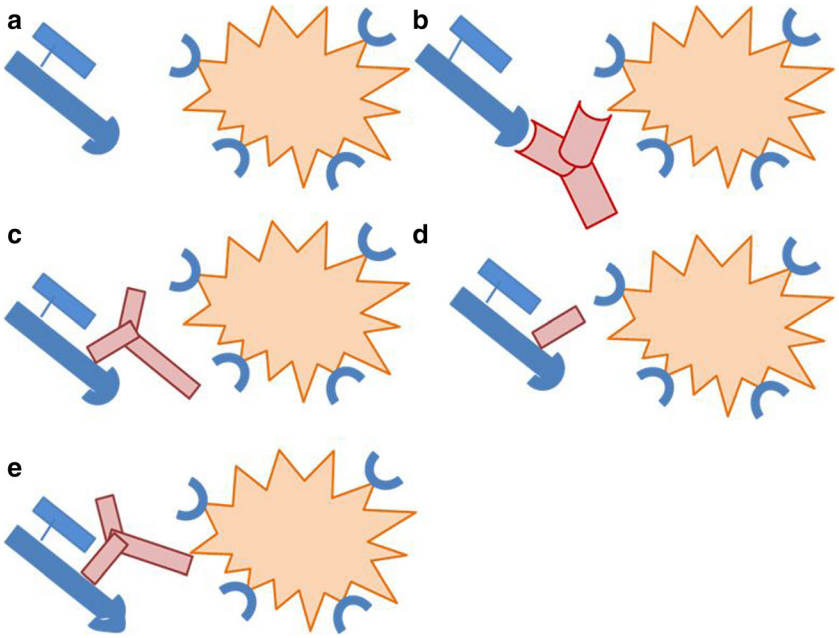

Fig. 2 Prevention of tetanus toxin binding to its receptors on the neuron by monoclonal antibodies. Tetanus toxin binds to its receptors on the neuron (**a**), binding of a monoclonal antibody to the receptor binding site of the toxin inhibits toxin's binding to the neuron (**b**) steric hindrance of the Fc region of a monoclonal antibody on the receptor binding site of the toxin results in the prevention of tetanus toxin binding to its receptors on the neuron (**c**), removing the Fc region of the monoclonal antibody leads to the loss of its neutralizing capacity in Fab fragment and subsequent toxin binding to its receptors (**d**), and conformational change of the receptor binding site of the toxin after binding of a monoclonal antibody to the toxin leads to the abolished binding of toxin to its receptors on the neuron (**e**)

protect the mice against tetanus; however, a combination consisted of anti-fragment B and anti-fragment C MAbs protected the mice against 7 lethal dose of the toxin (Ziegler-Heitbrock et al. 1986). Synergisms may also occur between two or more MAbs with neutralizing ability. Matsuda et al. showed that neutralizing capacity of a combination of three or five MAbs (43 IU/100 μg IgG), is 10-fold higher than that of the individual MAbs (4.3 IU/100 μg IgG). Furthermore, the protective activity of a combination of these MAbs was higher than that of the human polyclonal anti-tetanus immunoglobulin (43 IU/100 μg IgG for MAbs versus 33 IU/100 μg IgG for polyclonal anti-tetanus immunoglobulin). Additionally, these MAbs recognized epitopes located on different domains of tetanus toxin including fragment B, C in heavy chain and fragment A in light chain (Kamei et al. 1990).

Volk et al. clearly demonstrated that various combinations of MAbs may induce synergistic effects. They showed that a mixture composed of eight MAbs obtained from the spleen of mice immunized with fragment C, provided protection against tetanus in the mice at a concentration of 0.32 μg/ml for each antibody, while none of the individual MAbs were able to provide protection except one MAb, which neutralized the toxin at the concentration of 3.2 μg/ml. Furthermore, while none of 5 MAbs obtained from fragment B-immunized mice was protective, a mixture consisting of all 5 MAbs provided protection. The total required amount of neutralizing MAbs for toxin neutralization was reduced from 0.32 μg/ml or 3.2 μg/ml of a single MAb to 0.064 μg/ml, 0.017 μg/ml, and 0.013 μg/ml in the combinations consisted of 2, 3, and 4 MAbs, respectively. Toxin neutralizing activity of these mixtures was comparable to that of polyclonal immunoglobulin (15 to 77 IU/mg of protein for MAbs versus 29.5 IU/mg of protein for the polyclonal anti-tetanus antibodies). These results may suggest that an increase in the number of MAbs in a combination would improve the protective activity of the MAbs (Volk et al. 1984). Furthermore, the synergy could occur among MAbs recognizing epitopes located on the same domain of the toxin as well as those directed against different domains including fragment A, B, or C. Results of the study performed in our lab revealed a minimum of 1.25 μg of MAb 1F2C2 in combination with MAb 1F3E3 (1.25 μg) completely protected mice against 10 MLD of the toxin similar to the protection obtained with 10 μg of MAb 1F2C2 alone. MAb 1F3E3 did not neutralize the toxin in vivo even at the highest dose (10 μg). Results of competition ELISA showed that 1F2C2 and 1F3E3 recognize two distinct, but spatially or linearly very close or overlapping epitopes located within fragment C of tetanus toxin (Yousefi et al. 2014a; Yousefi et al. 2014b).

The same cooperative effect was observed among MAbs recognizing different epitopes of fragment A in the light chain. A combination of two fragment A-specific MAbs designated ST11 and ST15, which individually gave partial protection up to 6 days of tetanus toxin challenge, was able to completely

protect animals for more than 28 days after tetanus toxin challenge. The synergy was also observed among MAbs which individually fully protected the mice against tetanus toxin. The combinations consisted of MAb ST12, which individually was fully protective against tetanus toxin with a potency value of 13.5 IU/100 μg IgG, with the other fully protective MAbs including MAb ST15 (potency value of 10.5 IU/100 μg IgG) or MAb ST11 (potency value of < 0.5 IU/100 μg IgG) resulted in a significant increase in the potency value, up to 43 IU/100 μg IgG. This value was far higher than that of polyclonal anti-tetanus immunoglobulin, which was 0.263 IU/100 μg IgG. However, it is notable that not all combinations of anti-tetanus MAbs are able to fully protect the animals against toxin challenge, as evidenced by utilization of a combination consisted of two partial protective anti-tetanus MAbs, MAb ST15 and MAb ST17. This combination only gave a partial protection in the animals following tetanus toxin challenge (Lang et al. 1993).

Three mechanisms may account for the synergistic effect of a combination of MAbs (Volk et al. 1984; Diamant et al. 2015): (1) binding of one MAb may result in enhancement of the affinity of another MAb leading to a more effective neutralization of the toxin; (2) clearance of antibody-toxin immune-complexes is facilitated, since larger immune-complexes which are formed by binding of two or more MAbs to the toxin, will be removed faster than the smaller complexes from the circulation by phagocytosis; (3) simultaneous binding of MAbs directed against different functional epitopes of the toxin will result in a significant blockade of several steps which are crucial for cytotoxicity of tetanus toxin.

Determining Potency Value of MAbs

Replacement of current human polyclonal anti-tetanus immunoglobulin with a single or a combination of MAbs is feasible only if the MAbs or their combination confer protection against tetanus with a potency comparable to that of the polyclonal pool. However, determining potency value of MAbs by the standard method is questionable. Classically, the potency of an anti-tetanus preparation is determined by comparing the quantity of antibody necessary to protect mice against paralysis or death induced by a fixed quantity of tetanus toxin in a limited time (4 days), with the quantity of a reference preparation, which is a human polyclonal tetanus immunoglobulin that gives the same level of protection (Council of Europe 2010).

It has been shown that the kinetic pattern of dose-response of MAbs is different from that of the human polyclonal anti-tetanus antibodies. Ahnert-Hilger et al. reported that a mouse polyclonal anti-tetanus antibody irreversibly neutralized tetanus toxin in a dose-dependent manner within 4 days, while a mouse MAb was only able to delay the death of the animals (more than 4 days) and all the animals died at the end of the experiment (Ahnert-higer et al. 1983). The same phenomenon which is designated “delayed intoxication” was observed by Matusuda et al. They showed that using the MAbs in a dose below the minimum survival dose, resulted in a delay in the death of the mice intoxicated with tetanus toxin and finally all the animals died by the end of the study (Matsuda et al. 1992).

Comparison of potency values of the MAbs using the standard method with the method in which the treated mice were monitored for 20 days demonstrated an overestimation in the potency value of the MAbs between 1- and 30-fold (Matsuda et al. 1992). These findings indicate that the standard method for determining potency value of a polyclonal anti-tetanus immunoglobulin preparation with the observation period of 4 days is not applicable to the MAbs and treated animals must be monitored for much longer time (Matsuda et al. 1992). Accordingly, potency of the anti-tetanus MAbs should be determined based on a minimum survival dose which is required for protection of the animals against a fixed amount of tetanus toxin for at least 20 days (Matsuda et al. 1992).

Protective Epitopes of Tetanus Toxin as a Vaccine Candidate

Monoclonal antibodies are useful tools in identifying immunogenic and protective epitopes of the toxin for development of epitope-based vaccines. In order to identify the immunogenic epitopes located on the carboxyl terminal region of tetanus toxin (TTC), which is responsible for the binding of toxin to its receptors, various anti-tetanus MAbs were generated by Luo et al. and their neutralizing capacities were investigated. The highest level of protection against L+/10 dose of toxin was obtained with MAb 5C4. It was the only anti-fragment C MAb which was capable of blocking the binding of recombinant fragment C to GT1b gangliosides. Afterwards, epitope mapping of MAb 5C4 was performed using various recombinant derivatives of fragment C including $TTC_{(\text{amino acid (aa) } 865–1315)}$, $TC1_{(\text{aa } 865–975)}$, $TC2_{(\text{aa } 967–1154)}$, $TC3_{(\text{aa } 1155–1315)}$, $TC4_{(\text{aa } 1118–1315)}$, $TC5_{(\text{aa } 1042–1171)}$, and $TC6_{(\text{aa } 1172–1315)}$. MAb 5C4 recognized $TTC_{(865–1315)}$, $TC3_{(1155–1315)}$, $TC4_{(1118–1315)}$, and $TC5_{(1042–1171)}$, but did not bind to $TC1_{(865–975)}$, $TC2_{(967–1154)}$, and $TC6_{(1172–1315)}$. These results indicated that the targeted epitope of 5C4 is located in a region spanning residues Lys1155 to Val1171 ($TC_{(\text{aa } 1155–1171)}$). Thereafter, a peptide spanning Lys1155 to Val1171 of tetanus toxin was synthesized and conjugated with immunogenic carrier protein keyhole limpet hemocyanin (KLH) and then tested as a peptide-based vaccine against tetanus. According to the results, 100 μg of $TC_{(\text{aa } 1155–1171)}$-KLH in immunized mice led to 80% protection against a subsequent challenge with 100% lethal dose of the toxin (LD100%). Vaccination with 100 μg of recombinant fragment C and tetanus toxoid resulted in 100% protection of the mice. Accordingly, less titer of anti-

$TC_{(1155–1171)}$, anti-fragment C, and anti-toxin antibodies in $TC_{(1155–1171)}$-KLH immunized mice were generated compared with the mice that have been immunized with recombinant fragment C or the toxoid. It is notable that 80% protection against the tetanus toxin is not ideal, but it is acceptable for an epitope-based vaccine. Six other MAbs generated in this study also afforded protection against tetanus in the animals, with neutralizing activities ranged from 12.5 to 15.6 IU/mg, which were higher than that of the standard human polyclonal tetanus immunoglobulin (1.5 IU/mg). Their epitope mapping demonstrated that while three of these MAbs, including 3A6, 1A12, and 1H4 recognized fragment C, none of them were able to abolish binding of fragment C to GT1b gangliosides.This suggests that other receptors except GT1b gangliosides might be involved in binding of tetanus toxin to the neurons. It is also likely that binding of MAbs to the toxin leads to formation of immune-complexes which are removed from the circulation by Fc-receptor-mediated phagocytosis (Luo et al. 2012). In another study, efficacy of a fragment C-vaccine was compared to the equivalent dose of toxoid-vaccine. The fragment C-vaccine resulted in the complete protection of the vaccinated animals, while toxoid-vaccine only provided a partial protection against a subcutaneous challenge of 100,000-fold of LD50 in the animals (Yu et al. 2011; Fairweather et al. 1987). Fairweather and colleagues also showed that a vaccine consisted of the purified fragment C alone was as efficient as a vaccine composed of the whole recombinant fragment C together with 121 residues of fragment B (residues 743 to 1314) in eliciting a comparable level of antibody and protection against tetanus toxin (Fairweather et al. 1987). Results from another study showed that some derivatives of fragment C (TetC), including TetC1-451, TetC1-271, TetC1-180, and TetC80-451 were able to protect the animals against tetanus toxin challenge (Figueiredo et al. 1995) suggesting that a subunit vaccine spanning TetC80-180 of tetanus toxin might provide protection against tetanus.

Conclusion

Passive immunotherapy of tetanus with human polyclonal anti-tetanus immunoglobulin has many disadvantages including high cost, lot-to-lot heterogeneity, and the adverse reactions to the plasma proteins. Therefore, anti-tetanus MAbs which lack these drawbacks and show protective capacity against tetanus toxin might be considered as an alternative for passive immunotherapy against tetanus. Epitope mapping of MAbs with protective capacity against tetanus toxin has shown that protective epitopes of tetanus toxin are predominantly located on fragment C; nevertheless, the other two functional domains of the toxin including fragment A on the light chain and fragment B on the heavy chain may display neutralizing epitopes. Furthermore, utilization of a combination of anti-tetanus MAbs may dramatically increase the protective activity of the MAbs leading to a more effective immunotherapeutic activity. A cocktail of such MAbs may prove to be more effective and suitable than the currently available polyclonal anti-tetanus toxin preparations.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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