Methods for the Functional *In Vitro* Characterization of Tetanus Neurotoxin and Toxoids

Heike A. Behrensdorf-Nicol, Ursula Bonifas, Lena Böhl, Beate Krämer, and Karin Weisser

Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicals, Langen, Germany

Summary

Tetanus neurotoxin (TeNT) consists of two protein subunits: The heavy chain binds to neurons, whereas the light chain cleaves the protein synaptobrevin. Inactivated TeNT (tetanus toxoid) is used for vaccine production. In order to exclude residual toxicity, animal tests are prescribed by the European Pharmacopoeia for each toxoid bulk. Our aim is to replace or reduce these tests by providing in vitro methods for the detection and functional characterization of TeNT. The activities of the toxin subunits can be separately examined in a binding test and an endopeptidase assay. In addition, a combined assay takes into account both the receptor binding and the protease activity, and thus more closely mimics an in vivo infection. This set of in vitro methods allows more detailed measurements of the functional integrity of TeNT and toxoid preparations than the corresponding animal toxicity test does. These methods may be valuable tools for consistency monitoring during vaccine production, thereby helping to reduce toxicity testing in animals.

Keywords: Tetanus neurotoxin, toxoids, synaptobrevin, ganglioside GT1b

1 Introduction

Testing for tetanus toxicity

This article describes our activities on the *in vitro* detection and characterization of tetanus neurotoxin (TeNT). Tests for tetanus toxicity are mainly important for the production of tetanus vaccines. A crucial step in this process is the detoxification of TeNT by formaldehyde. According to the European Pharmacopoeia (EP), the resulting toxoid has to be tested for the absence of residual toxin and for irreversibility before it can be used in vaccines for veterinary or human use (Council of Europe, 2011a,b). For this test, a ten-fold vaccine dose of toxoid has to be injected into guinea pigs. Five of these animals receive toxoid which has been stored at 5°C for six weeks, and five others receive material which has been stored at 37°C. If no animal develops tetanus symptoms, the toxoid is regarded as safe. Our aim is to provide an *in vitro* method which should be able to replace these animal tests, or to reduce the number of animals needed. There are two important requirements for a replacement method: The detection limit has to be at least equivalent to the animal test; and the specificity (i.e., the ability to discriminate between toxic and nontoxic molecules) must be sufficient.

Mode of action of TeNT

Each TeNT molecule consists of two protein chains which are linked by a disulfide bond. The heavy (H-)chain (100 kDa) mediates binding to neurons and translocation of the toxin's light (L-)chain (50 kDa) into the cytoplasm of the target cell. During this translocation, both chains are separated by reduction of the disulfide bond. This leads to activation of the protease domain in the L-chain, which can then cleave the protein synaptobrevin. Synaptobrevin is present on the surface of neuronal vesicles and plays a crucial role in the release of neurotransmitters: When a neuron is activated, the synaptobrevin on the vesicles comes into contact with two proteins on the inner surface of the cell membrane named syntaxin and SNAP25. Together, these proteins form the SNARE complex. The formation of this complex precedes the fusion of the vesicle membrane with the cell membrane, which is required for neurotransmitter release. In the presence of the TeNT L-chain, however, synaptobrevin is cleaved, and the release of the neurotransmitters is blocked. As TeNT acts specifically in inhibitory interneurons, the blockade of signal transmission causes a severe spastic paralysis which is characteristic for tetanus infection.

Functional characterization of the toxin subunits

Our initial strategy was to detect active TeNT via its proteolytic activity. We have, therefore, developed an endopeptidase assay in which the reduced sample material is added to immobilized recombinant synaptobrevin on a microtiter plate (Kegel et al., 2007; Behrensdorf-Nicol et al., 2008). The synaptobrevin is cleaved by active TeNT, and the resulting fragment is detected using an antibody followed by a biotinylated secondary antibody, streptavidin-peroxidase, and a colorimetric substrate. This method is able to sensitively detect active TeNT L-chains.

We have also established an assay for the receptor-binding capacity of TeNT (Behrensdorf-Nicol et al., 2010). In this assay, the samples are incubated on a microtiter plate containing immobilized ganglioside GT1b, which is the receptor for TeNT. The detection of bound toxin is then performed with a tetanus

antiserum, again followed by a biotinylated secondary antibody, streptavidin-peroxidase, and a colorimetric substrate. This assay is able to detect functional TeNT H-chains.

When establishing these methods, we had hoped to find a reliable correlation between the binding capacity or the enzymatic activity of a sample and its *in vivo* toxicity. But when we tested tetanus toxoids from various vaccine manufacturers, we found that all tested batches were able to bind ganglioside GT1b to a varying extent. Moreover, most toxoids also showed high specific synaptobrevin-cleaving activities (Behrensdorf-Nicol et al., 2010; Behrensdorf-Nicol et al., 2008). However, all tested toxoids had already passed the mandatory animal test for absence of toxicity – and thus it appears that assays which measure only one activity (cleavage or binding) are not sufficient to reliably predict the *in vivo* toxicity of a sample. One major reason is that these assays are easily disturbed by free toxin subunits: Free TeNT L-chains can cleave synaptobrevin – and will thus generate signals in the endopeptidase assay. *In vivo*, however, they are nontoxic because they cannot bind to their target cells. Similarly, free H-chains are able to bind the tetanus receptor, but they cannot cleave synaptobrevin, and therefore are nontoxic. But although these single assays give no reliable information on the toxicity of a sample, they can nevertheless be valuable tools for specifically assessing the integrity of the heavy or light TeNT subunits in toxin and toxoid samples (for example, in the context of consistency monitoring).

2 Combined assay

In order to be toxic *in vivo*, a TeNT molecule must be able to bind its receptor, and, in addition, the same molecule must show enzymatic activity. This led to our new approach: A combined assay which takes into account the binding as well as the endopeptidase activity (Behrensdorf-Nicol et al., 2010). In the first step of this assay, intact TeNT molecules present in a sample bind to a ganglioside-coated plate via their H-chains, and unbound molecules are washed away. In the second step, the toxin's L-chains are released from the bound toxin molecules by reduction of the disulfide bridges. They are then transferred to a second plate, which is coated with synaptobrevin. Active L-chains will cleave the synaptobrevin, and the cleavage fragment is finally detected in an antibody-mediated reaction. Accordingly, this combined assay only detects TeNT molecules which possess functional binding and protease domains. Additionally, both domains must be present on different subunits which can be separated by reduction. Thus, the combined assay mimics a real infection much more closely than the single assays.

It could be shown (Behrensdorf-Nicol et al., 2010) that tetanus toxoids from the routine vaccine manufacturing process (which had passed the prescribed animal safety tests) generated only very low signals in the combined assay; in all cases the absorption values were much lower than the corresponding signals in the single assays. Moreover, when toxoids from different manufacturers were diluted to a fixed concentration (5 Lf/ml) and spiked with TeNT, the spiked toxin could be reliably detected by the combined assay. These observations showed that the combined assay results correlate more closely with the toxicity of a sample than the results of the single assays. But those studies also indicated that toxoids from different manufacturers varied in their behavior in the combined assay – while some toxoids did not induce any signals at all, others induced assay signals when tested in increasing concentrations. Also, the detection limit of the combined assay had not reached the limit of the *in vivo* toxicity test until recently. To address these shortcomings, further optimization studies were performed.

Enhancement of the enzymatic activity

Recently, a stimulating effect of trimethylamine N-oxide (TMAO) on the proteolytic activity of botulinum neurotoxins was described (Nuss et al., 2010). TMAO is a natural compound that helps cells withstand osmotic stress, and, in addition, is thought to stabilize proteins. With regard to the high similarities between botulinum and tetanus toxins, we tested whether this substance also has an effect on TeNT (Fig. 1). When TMAO was added to the endopeptidase assay it caused a strong enhancement of the cleavage signals. The maximal effect was obtained with a TMAO concentration of about 1 M. Also, when added to the cleavage step of the combined assay, TMAO strongly enhanced the toxin signals. The detection limit of the combined assay has been markedly improved by this finding. Although an exact determination has not yet been performed, for pure toxin samples it is thought to be close to the current animal test. The TMAO-induced enhancement of the toxin's proteolytic activity, however, also led to a further increase of the background signals obtained with some toxoids.

Optimization of the binding step

The best characterized receptor for TeNT is the ganglioside GT1b, a small glycolipid. The existence of an additional protein receptor is suspected (Yeh et al., 2010; Herreros et al., 2000), but not yet proven. The ganglioside GT1b consists of a hydrophilic carbohydrate part, which contains the toxin binding site and various other functional groups. The hydrophobic lipid part of GT1b, in contrast, is unreactive and can only undergo weak interactions. In order to further optimize the binding step of the combined assay, we have compared several strategies for the immobilization of the ganglioside on microtiter plates. An ideal protocol, however, has not yet been identified. When protocols for a passive immobilization were used, the GT1b molecules did not firmly attach to the plastic surface and could be easily washed away. When, in contrast, the functional groups in the hydrophilic part of GT1b were used to obtain a covalent immobilization, this usually led to low TeNT binding signals – presumably because the toxin binding site was either destroyed or not accessible. We are currently using a passive immobilization protocol as a compromise. A further critical point is that protein-glycolipid interactions are known to be strong and selective only if they are multivalent. In the case of TeNT, each molecule contains two binding sites which can simultaneously interact with gangliosides, and this bivalent interaction provides the basis for the strong binding of TeNT to neurons *in vivo* (Chen et al., 2009). So the question arises: Can a bivalent binding mode also be enhanced in our *in vitro* system, e.g., by

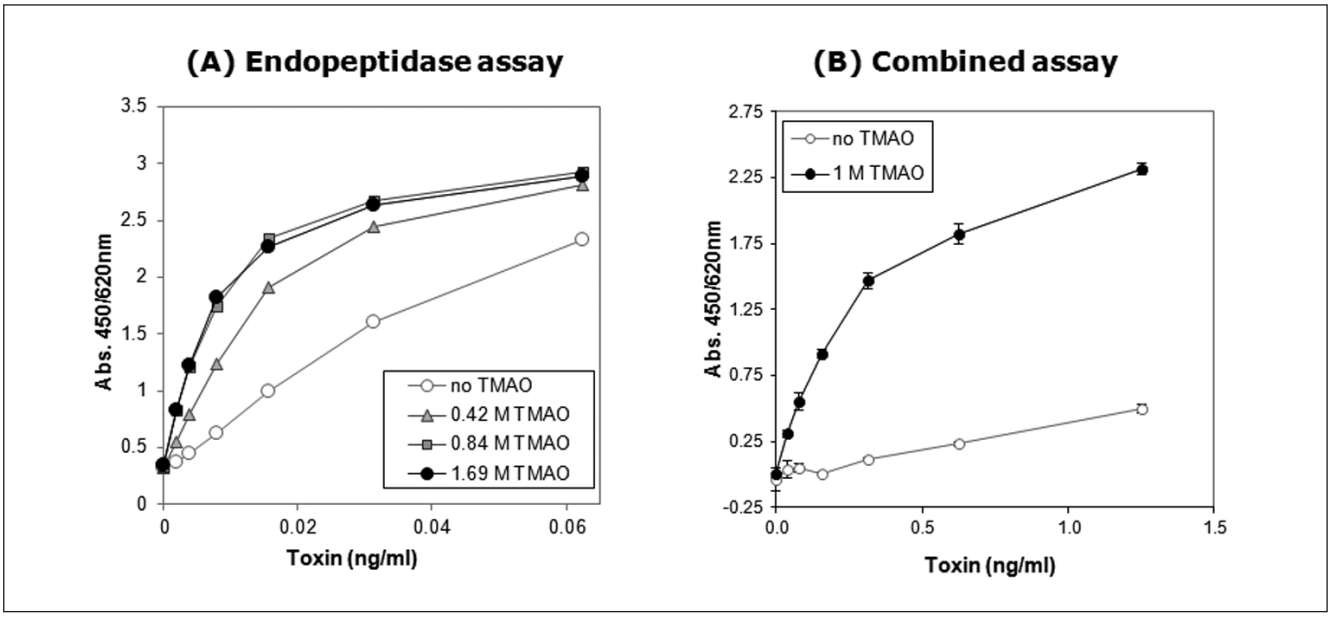

Fig. 1: Enhancement of the proteolytic activity by TMAO
The indicated concentrations of TeNT were analyzed in the endopeptidase assay (A) and in the combined assay (B). Both assays were performed as described previously (Behrensdorf-Nicol et al., 2010), with the exception that varying TMAO concentrations (0 M, 0.42 M, 0.84 M or 1.69 M TMAO in (A); 0 M, or 1.0 M TMAO in (B)) were used in the cleavage reaction.

using ganglioside mixtures containing the respective preferred receptor subtypes for both binding sites or by embedding the gangliosides into liposomes to increase their steric flexibility? These studies are currently ongoing.

Current status of combined assay
At the current stage of our project, the combined assay allows the function-based detection of TeNT with a detection limit for pure toxin samples that is presumably close to the animal test. However, before the combined assay can replace the *in vivo* toxicity tests, some obstacles have to be overcome. Most importantly, some toxoids still induce signals. These toxoid signals are much lower than in the single binding or cleavage tests, but nevertheless they show that the ability of the combined assay to discriminate between toxic and nontoxic molecules is insufficient. The formaldehyde-induced detoxification reaction is known to result in a large variety of molecular constructs, some of which are apparently still able to induce signals in the combined assay. For example, toxin molecules with an isolated defect in the translocation domain could still induce signals in the combined assay, but would not be toxic *in vivo*. So, the correlation between assay signal and toxicity may not be reliable under all circumstances. Unless this shortcoming is resolved, the combined assay alone may not prove to be an adequate replacement for the animal test.

3 Set of tools for toxin and toxoid characterization

Although the combined assay does not yet represent a reliable alternative to the animal toxicity test, we are nevertheless convinced that the methods developed in our laboratory provide a valuable set of tools for the characterization of TeNT preparations. They allow examination of different molecular aspects related to the toxin's *in vivo* function (Fig. 2). The binding test measures the presence of functional TeNT H-chains, the endopeptidase assay specifically quantifies active L-chains, and the combined assay takes into account the intactness of both subunits as well as their quaternary structure (i.e., their linkage by a disulfide bond). Thus, these methods can provide more relevant data concerning activity than physicochemical methods. They are also faster and, due to their ability to separately look at the single subunits, allow a more detailed functional characterization of toxin preparations than the corresponding animal tests (which only deliver a yes/no-answer). The assays could be useful, e.g., for monitoring the activity and consistency of toxin or toxoid batches during and after production. Thus, they might contribute to reducing the need for final product testing on animals.

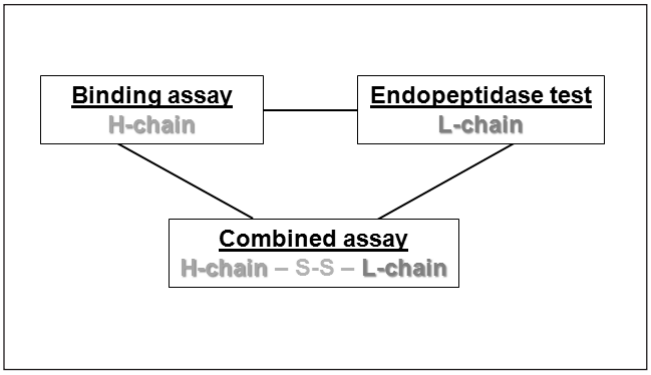

Fig. 2: Three assays as a set of tools for function-based TeNT characterization

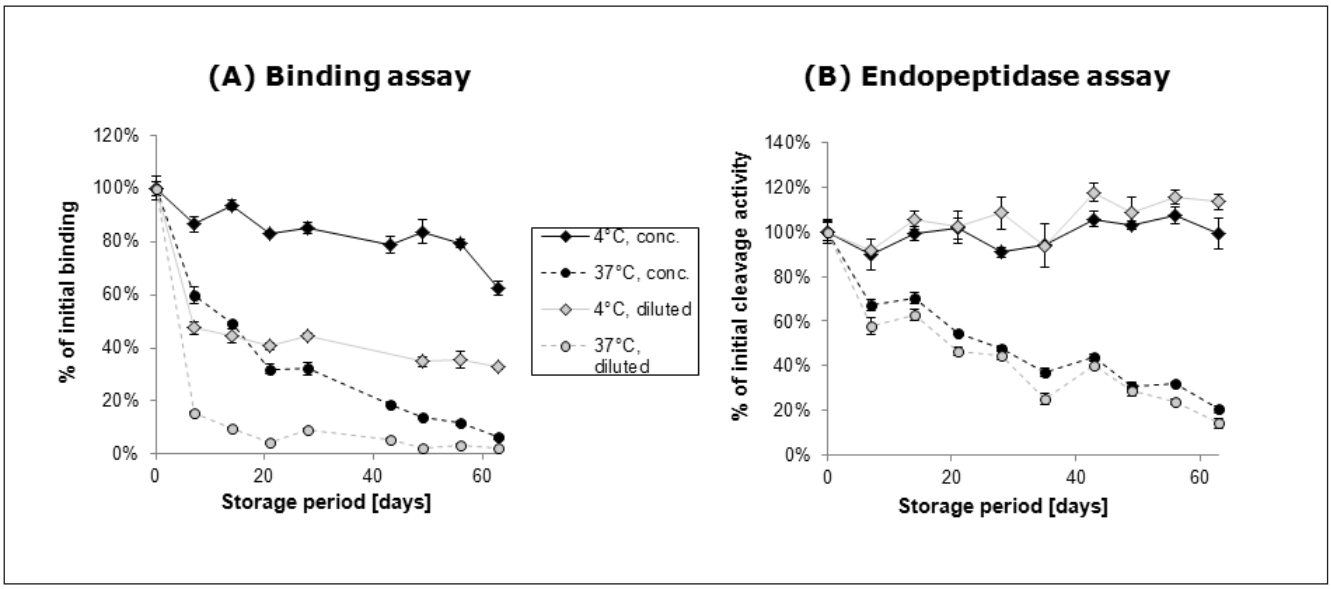

Fig. 3: Monitoring the stability of TeNT preparations

Crude TeNT solutions were stored over a period of nine weeks at 4°C (continuous lines) or 37°C (dotted lines) in either concentrated (60 µg protein/ml, black symbols) or diluted (6 µg/ml, grey symbols) form. The functional integrity of the stored material was tested weekly in the binding (A) and in the endopeptidase assay (B), which were performed as described previously (Behrensdorf-Nicol et al., 2010).

Application example 1

In order to check the applicability of this set of methods for monitoring functional changes in toxin or toxoid preparations, we have tested the stability of crude TeNT solutions over a storage period of nine weeks in the binding test and the endopeptidase assay (Fig. 3). A marked influence of the storage temperature on the toxin activity was clearly reflected in both assays – when stored at 4°C, the enzymatic activities of the TeNT L-chains remained constant over the complete 9-week period, while the activities of samples stored at 37°C continuously decreased. The binding capacities of the H-chains also decreased much faster at 37°C than at 4°C. We further examined the dependence of toxin stability on the protein concentration of the stored solutions. We found that the concentration during storage had no influence on the enzymatic activity of the TeNT L-chains. The H-chain, in contrast, was clearly influenced by the protein concentration; the binding capacities of the diluted samples showed a much steeper decline than those of the concentrated samples. So, in contrast to animal tests, which only show if a sample is still toxic or not, the set of *in vitro* methods allows a more detailed assessment of the changes in the respective toxin subunits.

Application example 2

The applicability of the *in vitro* assays for the functional quantification of tetanus antibodies has also been investigated. For this study, we pre-incubated TeNT with different dilutions of a tetanus antiserum or a control serum. Then the antibody-mediated neutralization of toxin activity was measured in the binding test and the endopeptidase assay (Fig. 4). The tetanus antiserum was able to inhibit the toxin's receptor binding capacity when applied in concentrations of 10^{-3} IU/ml or higher, and the proteolytic activity was blocked by concentrations starting from 10^{-4} IU/ml. With corresponding dilutions of a pre-immune serum, a weak inhibition was only obtained with the highest serum concentration. These data indicate that the described methods could be useful for the characterization and titer determination of tetanus antisera or immunoglobulin preparations. An advantage over other widely used methods – like the toxin binding inhibition (ToBI) test or enzyme-linked immunosorbent assays (ELISA) – would be that these tests measure the functional inhibition caused by the antibodies rather than simple binding to toxin. Accordingly, the results might be expected to better correlate with the actual protection *in vivo*.

4 Conclusion

We have shown that our combined assay is able to detect active TeNT on a functional basis with a similar sensitivity as the *in vivo* toxicity test in guinea pigs. The combined assay offers a much higher specificity than the single binding or cleavage assays, but its ability to discriminate between toxic and non-toxic molecules in the presence of toxoids is not yet sufficient. Before a decision can be taken as to whether the combined approach can replace the corresponding *in vivo* toxicity tests, further optimization is needed. We must also determine applicability to toxoids from all relevant manufacturers. Whatever the outcome of these studies, the function-based assays described herein can serve as valuable tools for consistency

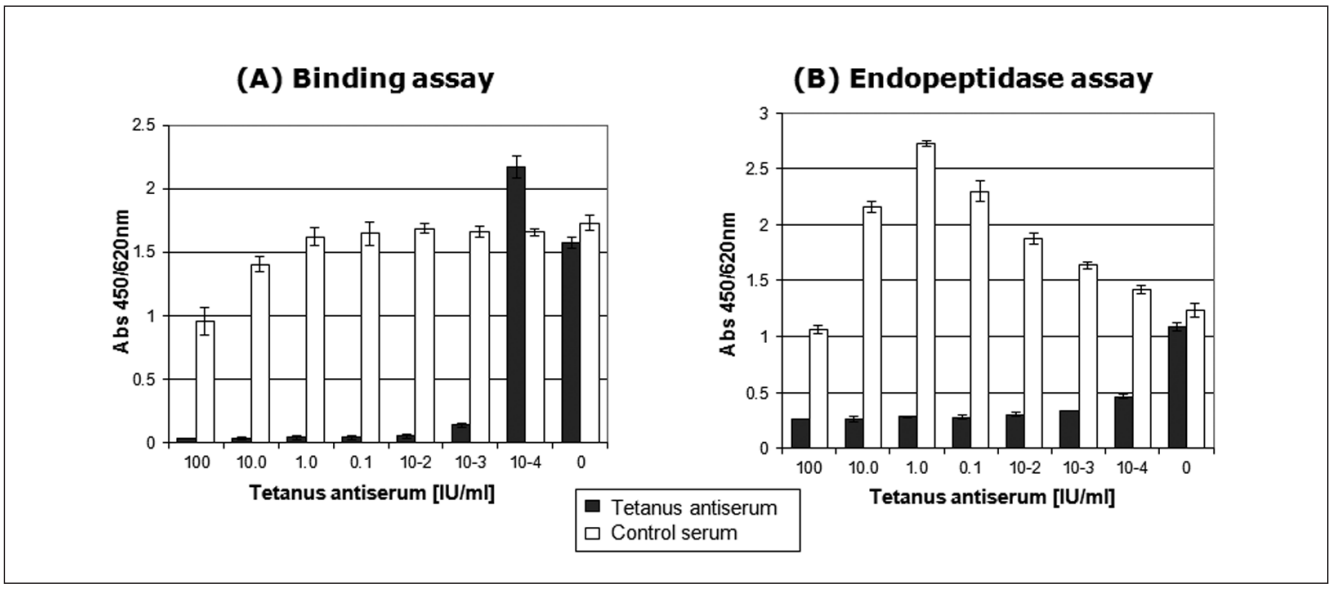

Fig. 4: Characterization of antisera

TeNT was pre-incubated with different dilutions of a tetanus antiserum (black columns) or a control serum (white columns). Then the neutralization of toxin activity was measured in the binding (A) and endopeptidase (B) assays, which were performed as described previously (Behrensdorf-Nicol et al., 2010).

measurements – and thus could also contribute to a reduction of animal testing. Furthermore, the potential applicability of the assays for the quantification of neutralizing antibodies has been demonstrated.

References

Behrensdorf-Nicol, H. A., Kegel, B., Bonifas, U., et al. (2008). Residual enzymatic activity of the tetanus toxin light chain present in tetanus toxoid batches used for vaccine production. *Vaccine 26,* 3835-3841.

Behrensdorf-Nicol, H. A., Bonifas, U., Kegel, B., et al. (2010). In vitro determination of tetanus toxicity by an endopeptidase assay linked to a ganglioside-binding step. *Toxicol. In Vitro 24*, 988-994.

Chen, C., Fu, Z., Kim, J. J., et al. (2009). Gangliosides as high affinity receptors for tetanus neurotoxin. *J. Biol. Chem. 284*, 26569-26577.

Council of Europe (2011a). Tetanus vaccine (adsorbed), monograph 0452. In *European Pharmacopoeia*, 7th edition.

Council of Europe (2011b). Tetanus vaccine for veterinary use, monograph 0697. In *European Pharmacopoeia*, 7th edition.

Herreros, J., Lalli, G., Montecucco, C., and Schiavo, G. (2000). Tetanus toxin fragment C binds to a protein present in neuronal cell lines and motoneurons. *J. Neurochem. 74*, 1941-1950.

Kegel, B., Behrensdorf-Nicol, H. A., Bonifas, U., et al. (2007). An in vitro assay for detection of tetanus neurotoxin activity: Using antibodies for recognizing the proteolytically generated cleavage product. *Toxicol. In Vitro 21*, 1641-1649.

Nuss, J. E., Wanner, L. M., Tressler, L. E., and Bavari, S. (2010). The osmolyte trimethylamine N-oxide (TMAO) increases the proteolytic activity of botulinum neurotoxin light chains A, B, and E: implications for enhancing analytical assay sensitivity. *J. Biomol. Screen. 15*, 928-936.

Yeh, F. L., Dong, M., Yao, J., et al. (2010). SV2 mediates entry of tetanus neurotoxin into central neurons. *PLoS Pathog. 6*, e1001207.

Acknowledgements

We thank Birgit Kegel, Katja Silberbach, Karin Duchow, and Manfred Moos from the Paul-Ehrlich-Institute and Thomas Binz from the Medical School Hannover for their support. And we would like to thank those who funded the project: The Doerenkamp-Zbinden Foundation, Animalfree Research, the Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing (SET), and the German Federal Research Department (BMBF).

Correspondence to

Heike Behrensdorf-Nicol, PhD
Department of Veterinary Medicine
Paul-Ehrlich-Institut (Federal Institute for Vaccines and Biomedicals)
Paul-Ehrlich-Strasse 51-59
63225 Langen, Germany
Phone: +49 6103 777416
Fax: +49 6103 771254
e-mail: Heike.Behrensdorf@pei.de