Review Article

"BINACLE" Assay for *In Vitro* Detection of Active Tetanus Neurotoxin in Toxoids

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Summary

Tetanus neurotoxin (TeNT) consists of two protein chains connected by a disulfide bond: The heavy chain mediates the toxin binding and uptake by neurons, whereas the light chain cleaves synaptobrevin and thus blocks neurotransmitter release. Chemically inactivated TeNT (tetanus toxoid) is utilized for the production of tetanus vaccines. For safety reasons, each toxoid bulk has to be tested for the "absence of toxin and irreversibility of toxoid." To date, these mandatory toxicity tests are performed in guinea pigs. A replacement by an animal-free method for the detection of TeNT would be desirable.

The BINACLE (BINding And CLEavage) assay takes into account the receptor-binding as well as the proteolytic characteristics of TeNT: The toxin is bound to immobilized receptor molecules, the light chains are then released by reduction and transferred to a microplate containing synaptobrevin, and the fragment resulting from TeNT-induced cleavage is detected by an antibody-mediated reaction. This assay offers a higher specificity for discriminating between toxic TeNT and inactivated toxoid molecules than other published assays.

Validation studies have shown that the BINACLE assay allows the sensitive and robust detection of TeNT in toxoids and thus may represent a suitable alternative to the prescribed animal safety tests for toxoids from several European vaccine manufacturers. Product-specific validations (and possibly adaptations) of the assay protocol will be required.

A European collaborative study is currently being initiated to further examine the applicability of the method for toxoid testing. The final aim is the inclusion of the method in the European Pharmacopoeia.

Keywords: tetanus neurotoxin, toxoids, synaptobrevin, ganglioside GT1b, *in vitro* toxicity assay

1 Introduction

This article describes the "BINACLE" (BINding And CLEavage) assay for *in vitro* detection of active tetanus neurotoxin. It provides an overview of the current state of assay validation and highlights the potential applicability of this new assay for the safety and consistency testing of tetanus vaccines. With regard to safety testing, the assay may represent an animal-free alternative to the mandatory guinea pig toxicity tests that are prescribed for tetanus vaccines to date. A replacement of these tests by an *in vitro* method would spare thousands of guinea pigs each year.

2 Tetanus neurotoxin (TeNT) and tetanus toxoids

TeNT, the neurotoxin produced by the ubiquitous bacterium *Clostridium tetani*, is one of the most potent toxins known. The estimated lethal dose for humans and many animal species is

below 2.5 ng per kg body weight (Gill, 1982). TeNT consists of two protein chains, which are connected by a disulfide bond. The mechanism of action is complex: The toxin first binds to receptors on motor neurons via its heavy chain (H-chain, 100 kDa). Ganglioside GT1b has been repeatedly described as the most important receptor for TeNT (e.g., by Chen et al., 2008; Binz and Rummel, 2009), although a recent report indicates that, in addition, the toxin may also use a protein receptor (Bercsenyi et al., 2014). After binding, the toxin is taken up by endocytosis and undergoes retrograde axonal transport to the central nervous system (Deinhardt et al., 2006). Here, the toxin is released and then enters an inhibitory interneuron by an endocytic mechanism. A pH-induced conformational change results in the insertion of the translocation domain (which is part of the H-chain) into the membrane of the endocytic vesicle. It thereby forms a transmembrane channel through which the toxin's light chain (L-chain, 50 kDa) is translocated into the cytosol (Pirazzini et al., 2013). The concomitant reduction of the inter-chain disulfide bond leads to the release of the L-chain as well as to the activa-

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tion of its metalloprotease domain, which specifically cleaves the neuronal protein synaptobrevin and thus inhibits neurotransmitter release (Link et al., 1992; Schiavo et al., 1992). As TeNT selectively acts in inhibitory interneurons, this blockade leads to severe spasms which are characteristic for tetanus disease.

Tetanus toxoids are preparations of TeNT that have been chemically inactivated. Following adsorption to a mineral adjuvant, these toxoids are utilized as tetanus vaccines for human or veterinary use. The inactivation is usually accomplished by treatment with formaldehyde, which causes unspecific cross-linking of the toxin molecules. Accordingly, the toxoids contain a large variety of molecular constructs bearing diverse intra- or inter-molecular crosslinks. These formaldehyde-induced modifications are thought to directly block or impede the native function of the involved protein domains (like, e.g., the receptor binding or the proteolytic domain). Besides, inter-chain crosslinks may additionally contribute to detoxification by preventing the release of the light toxin chain into the neuronal cytosol (Thaysen-Andersen et al., 2007).

3 Animal safety tests mandatory for tetanus toxoids

For safety reasons, each toxoid bulk has to be tested for the “absence of toxin and irreversibility of toxoid” according to the instructions of the European Pharmacopoeia before it can be used in vaccines for human or veterinary use (Council of Europe, 2014a,b). To date, these tests have to be performed as *in vivo* toxicity tests in guinea pigs: In order to prove the irreversibility of the inactivation, a ten-fold vaccine dose of non-adsorbed toxoid is injected into ten animals. Five of these receive toxoid that previously has been stored at 5°C for 6 weeks; the remaining five animals are injected with material that has been stored at 37°C. For batch testing of tetanus toxoids for human use, five additional guinea pigs are injected with a concentrated toxoid solution to prove the absence of any residual toxin. Accordingly, the testing of each bulk requires 10 to 15 guinea pigs in total. If none of the animals develops tetanus symptoms within 21 days after the injection, the toxoid bulk is regarded as safe. If the toxoid inactivation process has been sufficiently stringent, the test animals only experience low distress during these tests, which mainly results from the toxoid injection itself. If, however, a toxoid bulk has not been adequately inactivated, the animals suffer from tetanus symptoms, i.e., severe muscle spasms, which can finally lead to death by asphyxiation.

The total numbers of animals used for these safety tests are difficult to estimate, as no official data are available. But, taking into account the highly prevalent use of tetanus vaccines for the protection of humans and domestic animals world-wide, thousands of guinea pigs must be used annually for the safety testing of tetanus toxoids.

Besides the toxoid tests described above, the final tetanus vaccines have to undergo additional safety tests in some cases: During validation of the production method for human combination vaccines, tests for “specific toxicity of the tetanus component” in guinea pigs are required (e.g., Council of Europe,

2014c,d). For veterinary tetanus vaccines, a final product test for “residual toxicity” on guinea pigs is prescribed (Council of Europe, 2014b). As the final vaccines contain the toxoid in an adjuvant-adsorbed state (i.e., the toxoid molecules are not freely accessible for *in vitro* quantification methods), these safety tests are not within the scope of this review. The method described herein has been specially developed for use with non-adjuvanted toxoids as an alternative to the animal tests for the “absence of toxin and irreversibility of toxoid”.

4 Alternative assays for detection of tetanus toxicity

For animal welfare reasons, it would be highly preferable to replace the animal toxicity test by a suitable animal-free method. In addition, there are also prominent economic incentives for a replacement, as the animal test with its long observation period is cost-intensive and time-consuming.

Potential replacement methods have to be at least as sensitive as the animal test, i.e., already toxin concentrations in the low picomolar range must be detectable. Besides, suitable alternative methods must be able to reliably discriminate between active TeNT molecules capable of performing all actions leading to a toxic effect *in vivo* (i.e., receptor binding, translocation across the membrane and substrate cleavage) on the one hand and inactivated toxoid molecules (in which at least one relevant toxin function has been blocked) on the other hand.

Several types of *in vitro* assays for TeNT detection have been described to date: ELISA-based methods only detect the presence of toxin molecules without discriminating between active and inactivated toxin molecules, and therefore cannot measure the toxic activity of a sample. Cell-based assays, in contrast, are capable of discriminating between toxic and inactivated TeNT. However, they usually suffer from insufficient sensitivity (especially when using cell lines) or low robustness (particularly when using primary neuronal cells) (Sesardic, 2008).

Function-based biochemical assays also offer the potential to discriminate between active and inactive TeNT molecules. However, most biochemical methods described to date measure only one activity of the toxin. In particular, several endopeptidase assays have been published which detect active TeNT based on its synaptobrevin-cleaving activity (Kegel et al., 2007; Leung et al., 2002; Perpetuo et al., 2002). Such assays are usually simple, sensitive and fast, but suffer from a substantial drawback: Their results do not reliably correlate with the toxicity of a sample, as they only look at the functional capability of a single protein region (e.g., the protease domain), and do not consider the integrity of the remaining functional domains. Thus, these methods can easily be interfered with by fragmented or partly inactivated toxin molecules containing an intact protease domain but bearing defects in other relevant regions (e.g., the receptor binding site). Such molecules are not toxic *in vivo*, but would generate signals in an endopeptidase assay and thus cause false-positive results.

In particular, such “one-parameter assays” cannot reliably predict the *in vivo* toxicity of toxoid samples containing com-

Fig. 1: Principle of the BINACLE assay for detection of active TeNT

I) Functional TeNT molecules (light grey) bind to immobilized ganglioside GT1b (black) via their H-chains. Then the TeNT L-chains are released by reduction of the disulfide bonds. II) The supernatant containing the liberated L-chains is transferred to a plate containing the immobilized substrate protein synaptobrevin (dark grey). III) Functional TeNT L-chains cleave the synaptobrevin and the cleavage fragment is detected using a specific antibody followed by a biotinylated secondary antibody and streptavidin-peroxidase, which finally induces a color signal.

plex mixtures of toxin molecules that have been inactivated at different sites. It has been demonstrated that tetanus toxoids from relevant vaccine manufacturers retained high synaptobrevin-cleaving activities (Behrendorf-Nicol et al., 2008, 2010), although they had passed the mandatory manufacturer's animal tests for absence of toxicity. This finding illustrates the lacking correlation between the results from such *in vitro* assays and the actual toxicity of a toxoid sample.

5 The BINACLE concept

In order to be toxic *in vivo*, a TeNT molecule must both be able to bind its receptor and have proteolytic activity. Accordingly, assays for the reliable detection of tetanus toxicity should take into account the activities of more than one functional domain. This recommendation has also been made for *in vitro* detection methods of other bacterial toxins with similar modes of action, like botulinum or pertussis toxins (ICCVAM-NICEATM, 2008; Corbel and Xing, 2004). We have developed an assay format that takes into account the receptor-binding as well as the synaptobrevin-cleaving characteristics of active TeNT (Behrendorf-Nicol et al., 2010). This new method is called the BINACLE (BINDing And CLEavage) assay (Behrendorf-Nicol et al., 2013).

In the first step of the BINACLE assay (Fig. 1), active TeNT molecules bind via their H-chains to a microplate coated with the receptor molecule ganglioside GT1b. Then unbound molecules are washed away. By reduction of the disulfide bridges, the L-chains are then released from the bound H-chains and transferred to a second plate that is coated with the substrate protein synaptobrevin. Active L-chains cleave the synaptobrevin, and the cleavage fragment is detected by an antibody-mediated reaction leading to a colorimetric signal.

Accordingly, the BINACLE assay only detects TeNT molecules with an intact binding domain as well as a functional protease domain. As an additional requirement, both domains must be situated on different protein subunits which can be separated by reduction. Thus, the combined assay mimics the processes of a real infection much more closely than a pure cleavage test. We could show that this "combined" assay offers a much higher specificity for discriminating between toxic and detoxified TeNT preparations than the separate endopeptidase or binding assays (Behrendorf-Nicol et al., 2010). In 2010, this assay concept was awarded the 29th German Animal Protection Research Prize by the Federal Ministry of Food, Agriculture and Consumer Protection.

6 Status quo of the BINACLE assay

The replacement of a prescribed animal test by an alternative *in vitro* method is a complex procedure that can take many years. An overview of the fundamental steps related to the development, validation and implementation of the BINACLE assay is shown in Figure 2. Following comprehensive optimization (Behrendorf-Nicol et al., 2010, 2012), the finalized BINACLE assay protocol was subjected to an in-house validation study (Behrendorf-Nicol et al., 2013). This study showed that the BINACLE assay is able to detect active TeNT with a detection limit of approximately 0.03 ng/ml, which is comparable to the estimated detection limit of the *in vivo* test. The sensitive detection of toxoid samples spiked with active TeNT (in order to mimic insufficiently detoxified material) was also demonstrated. The precision of the method was shown to be satisfactory, with inter- and intra-assay variability both generally below 15%.

Fig. 2: Overview of the fundamental steps leading from the development towards the validation and prospective implementation of the BINACLE assay for the safety testing of tetanus toxoids

Moreover, the data obtained to date with toxoids from several European vaccine manufacturers indicate that the BINACLE assay may indeed be able to replace the prescribed animal safety tests for some of the most relevant products on the European market. The assay is able to sensitively detect active TeNT in these toxoids and, in addition, it is much faster than the animal test, and the methodological requirements with regard to skills and equipment are low.

However, the BINACLE assay might not allow sensitive toxicity testing in all cases: Toxoids from some manufacturers tend to generate concentration-dependent background signals in the BINACLE assay, even if they are sufficiently inactivated (Behrendorf-Nicol et al., 2013). This may result from the assay's inability to detect deactivation of the translocation domain: Toxin molecules with an isolated defect in this region could still induce signals in the BINACLE assay, while they would not be toxic *in vivo*. The degree of toxoid cross-linking is known to be determined by the formaldehyde concentration used for the inactivation (Thaysen-Andersen et al., 2007). Accordingly, the BINACLE assay may be best suited for testing toxoids from manufacturers which use particularly stringent inactivation protocols, resulting in toxoid molecules containing multiple inactivating crosslinks. Toxoids produced by less stringent detoxification protocols, in contrast, could contain a higher percentage of molecules bearing isolated defects in only one functional domain, and accordingly might be expected to generate higher intrinsic signals in the BINACLE assay. It will have to be determined whether the introduction of product-specific adaptations to the assay protocol may allow minimization of the background signals induced by some of the toxoids,

and thus may help to further increase the potential range of applicability of the method.

In order to further characterize the applicability of the BINACLE assay to vaccine testing, a study including four international laboratories has been performed to determine the lab-to-lab transferability of the assay protocol (Behrendorf-Nicol et al., 2014). This study has confirmed that the BINACLE assay is robust and easily transferable. Based on these promising results, the European Directorate for the Quality of Medicines and Healthcare (EDQM) has agreed to coordinate a European collaborative study via its Biological Standardization Program. Provided that the results of this study, which will be completed in 2015, further substantiate the applicability of the BINACLE assay to the safety testing of toxoids, we are intending to move on towards an inclusion of the method into the corresponding monographs of the European Pharmacopoeia (Council of Europe, 2014a,b) by submitting a "Request for Revision" to the European Pharmacopoeia Commission. This will be an important step towards promoting the rapid implementation and regulatory acceptance of the *in vitro* assay.

7 Applicability of the BINACLE assay for consistency testing

While BINACLE signals generated by toxoids from different manufacturers were found to differ markedly in some cases, batches from the same manufacturer generally showed a low batch-to-batch variation (Behrendorf-Nicol et al., 2013). Due to this, the BINACLE assay may represent a valuable tool for

monitoring the consistency of toxoid batches during vaccine production: Each manufacturer could define the typical signal range for his toxoids. When a new batch generates BINACLE signals that differ from this range, this would indicate that something in the production process might have gone wrong. This strategy is well in line with the consistency approach, a new trend in the quality control of vaccines that is based on a close monitoring of the whole production process by various *in vitro* methods in order to minimize variability between product batches. By using this strategy, the comprehensive *in vivo* testing of the final product will eventually become less important or even dispensable (De Mattia et al., 2011). In this context, the components of the BINACLE assay – i.e., the binding and the endopeptidase assay, which can be performed either separately or in combination – may help to characterize TeNT or tetanus toxoid preparations during vaccine production. These assays allow examining different molecular aspects related to the toxin's *in vivo* function: The binding test detects functional TeNT H-chains, the endopeptidase assay quantifies active L-chains, and the complete BINACLE assay takes into account the integrity of the whole toxin molecules. Together, these tools allow a more detailed functional characterization of toxin preparations than animal toxicity tests. By helping to monitor the activity and consistency of toxin or toxoid batches during vaccine production, these assays could further contribute to reducing the need for final product testing on animals.

8 Conclusion and future prospects

In conclusion, the validation results obtained to date have shown that the BINACLE assay may represent a suitable alternative to some of the prescribed animal tests for tetanus toxicity. However, as the applicability of the assay seems to depend on the toxoid production process, each manufacturer will have to validate the assay for his or her products by testing larger numbers of production batches (including “good” toxoid batches as well as insufficiently inactivated ones). The next step in the assay validation process is a European collaborative study which will be performed in order to further examine the applicability of the method for toxoid testing. The final aim is the inclusion of the method in the European Pharmacopoeia.

In addition, the BINACLE assay for the detection of active TeNT may also help vaccine manufacturers to monitor the consistency of their toxoid batches more closely and thus could further contribute to a reduced need for final product testing *in vivo*.

Further, we are currently examining whether the BINACLE approach can also be valuable for activity measurements of other bacterial toxins with a related molecular structure, like botulinum neurotoxins.

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Conflict of interest statement

All authors state that they have no potential conflicts of interest.

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