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In vitro determination of tetanus toxicity by an endopeptidase assay linked to a ganglioside-binding step

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ABSTRACT

Assays for the detection of tetanus neurotoxin (TeNT) are relevant for research applications as well as for the safety testing of tetanus vaccines. So far, these assays are usually performed as toxicity tests in guinea pigs or mice. The alternative methods described to date were mostly based on the detection of the toxin's proteolytic activity. However, these endopeptidase assays turned out to be unreliable because they only measure the enzymatic activity as sole determinant of tetanus toxicity, while not taking into account other parameters like the toxin's capacity to bind to target cells. In order to better reflect the *in vivo* situation of a tetanus infection, we have linked an endopeptidase assay to a ganglioside-binding step. The resulting method, which offers a unique combination of two functionally linked assays, detects those TeNT molecules only which possess both a functional binding domain as well as an active enzymatic domain. Our results demonstrate that this assay is able to reliably detect TeNT, and therefore might provide a basis for the replacement of the animal tests for detection of tetanus toxicity. Moreover, the assay concept could also be useful for *in vitro* toxicity measurements of other toxins with similar subunit structures.

1. Introduction

The characteristic symptoms associated with tetanus infections are induced by a toxin of the bacterium *Clostridium tetani*. This tetanus neurotoxin (TeNT) consists of two disulfide-linked subunits: the heavy (H-)chain (100 kDa) harbors the binding and translocation domains, and the light (L-)chain (50 kDa) contains a zinc-dependent protease domain. In the course of a tetanus infection, the H-chain binds to a motor neuron and mediates the internalization of the toxin molecule. Inside the motor neuron, TeNT is transported retroaxonally to the spinal cord, where it subsequently enters an inhibitory interneuron. In this target cell, the TeNT L-chain is separated from the H-chain and translocated into the cytosol. Due to the separation step, the protease domain of the L-chain is activated and finally cleaves the neuronal protein synaptobrevin. This protein, which is also called vesicle associated membrane protein (VAMP), plays an essential role in the release of neurotransmitters from the synaptic vesicles. Accordingly, the TeNT-induced cleavage of synaptobrevin results in a blockade of inhibitory neurotransmitter release and thus leads to the spastic paralysis which is characteristic of tetanus infections. With lethal doses below 2.5 ng per kg of body weight reported for humans and many animal species (Gill, 1982), TeNT is one of the most toxic substances known.

Reliable tests for tetanus toxicity are relevant for research applications as well as for the safety testing of vaccines. Tetanus vaccines are produced from TeNT which has been detoxified by formaldehyde. This treatment induces the formation of covalent intra- and intermolecular cross-links between the toxin molecules. The resulting inactivated product, which is referred to as toxoid, represents a heterogeneous mixture of protein complexes of varying sizes (Bolgiano et al., 2000). A key feature of these toxoids is that, while having lost their toxicity, they are still highly immunogenic. According to the specifications of the European Pharmacopoeia (EP) (Council of Europe, 2008a,b), each batch has to be tested for "absence of toxin and irreversibility of toxoid" before it can be used in vaccines for human or veterinary use. These tests are performed by injecting the toxoid material into guinea pigs and subsequently observing the animals for tetanus symptoms over a period of 21 days. At least 15 guinea pigs are required for the complete safety testing of each batch of tetanus toxoid. For research applications, in contrast, tetanus toxicity is frequently tested in mice (Robinson, 1988).

Abbreviations: BoNT, botulinum neurotoxin; BSA, bovine serum albumin; EP, European Pharmacopoeia; H-chain, heavy chain; L-chain, light chain; Lf, limit of flocculation; PBS, phosphate buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; rSyb2, recombinant synaptobrevin-2; TCEP, tris(2-carboxyethyl)-phosphine hydrochloride; TeNT, tetanus neurotoxin; TMB, 3,3′,5,5′-tetramethylbenzidine.

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In line with the "3R" concept of reducing, replacing or refining animal tests (Russell and Burch, 1959), efforts to develop a suitable *in vitro* method as alternative to these *in vivo* toxicity tests have been ongoing for some time. As a first step, we have developed an endopeptidase assay for the specific detection of TeNT based on its proteolytic activity (Kegel et al., 2002, 2007). For this assay, test samples are incubated with immobilized recombinant synaptobrevin, and the cleavage fragment which is generated in the presence of active TeNT is finally quantified with an antibody. This method has been shown to reach a detection limit close to the *in vivo* assay (Kegel et al., 2007). Similar, albeit less sensitive, endopeptidase assays for TeNT have also been described by other groups (Sesardic et al., 2000; Perpetuo et al., 2002; Leung et al., 2002). However, a major drawback of these endopeptidase assays is that they use enzymatic activity as sole indicator of toxicity, while all other essential steps of the *in vivo* intoxication process (namely binding, endocytosis, and entry of the toxin's L-chain into the cytosol) are not taken into account (Sesardic, 2008; ICCVAM-NICEATM, 2008). While it can be assumed that samples lacking endopeptidase activity almost certainly cannot exert any toxic effect, the converse is not necessarily true: it is possible that samples show proteolytic activity but lack *in vivo* toxicity (e.g. due to an impaired binding domain of the toxin molecules). Such samples would thus cause false-positive results in the endopeptidase assay. For example, we have demonstrated that many tetanus toxoids from the routine vaccine production process possess high residual endopeptidase activities (Behrensdorf-Nicol et al., 2008). These activities are presumably caused by functional TeNT L-chains which are not linked to intact H-chains and therefore do not exert any toxicity. The presence of residual enzymatic activities in sufficiently inactivated tetanus toxoids as well as in botulinum and pertussis toxoids has also been described by other groups (Sesardic, 2008; Gomez et al., 2007). These findings clearly demonstrate that endopeptidase assays alone are not sufficient for the reliable *in vitro* detection of tetanus toxicity.

Our aim was to develop an improved method which also takes into account additional molecular determinants of tetanus toxicity like the toxin's capacity to bind to target cells. Comparable concepts of an enzymatic assay performed in conjunction with a binding assay have previously been proposed for the detection of other bacterial toxins with similar molecular structures, like pertussis toxin (Corbel and Xing, 2004; Gomez et al., 2006) and botulinum neurotoxins (ICCVAM-NICEATM, 2008; Christian and Shine, 2008; Evans et al., 2009). But to our knowledge, no such assay has been published for TeNT so far. In this publication, we describe a combined assay for the detection of tetanus toxicity which consists of a ganglioside binding assay in combination with an endopeptidase assay measuring the synaptobrevin-cleaving activity. The two assays are functionally linked in such a way that only molecules which possess both binding and enzymatic activity on separable subunits finally generate a signal. We have investigated the capability of this combined assay to reliably detect TeNT in pure toxin solutions as well as in spiked toxoid samples.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich Chemie GmbH (Munich, Germany), if not otherwise stated. Protease-free bovine serum albumin (BSA) was obtained from Serva (Heidelberg, Germany); asolectin and PIPES were purchased from Fluka (Buchs, Switzerland). A 40 mg/ml stock solution of asolectin in PBS was prepared by sonication.

Pure TeNT was purchased from Sigma. For safety reasons, the toxin was handled in a microbiological safety cabinet by personnel who had been immunized against tetanus disease. Toxin-containing waste was inactivated by a treatment with 4% formaldehyde.

Samples of different tetanus toxoid batches were obtained from four European vaccine manufacturers (referred to as manufacturers A, B, C, and D). These batches had been taken from the normal vaccine production run and had passed the compulsory safety tests for "absence of toxin and irreversibility of toxoid" (Council of Europe, 2008a,b) performed by the manufacturers. The toxoid concentrations used in the respective experiments are indicated in Lf (limit of flocculation) per ml.

Recombinant synaptobrevin-2 (rSyb2) comprising the amino acids 1–97 of rat synaptobrevin-2 with an N-terminal histidine-tag was produced as described before (Kegel et al., 2007).

2.2. Binding assay

A MaxiSorp microtiter plate (Nunc, Langenselbold, Germany) was coated with 100 µl/well of a solution of ganglioside GT1b (Sigma) in methanol and allowed to dry. Unless otherwise indicated, a ganglioside concentration of 10 µg/ml was used for this step. Control wells were instead treated with methanol without added gangliosides. After washing four times with PBS/T (PBS/0.05% Tween 20), 250 µl PBS/5% sucrose/1% BSA/200 µg/ml asolectin were added to each well and incubated for 2 h at 37 °C in a microplate shaker. Then the plate was washed four times with PBS/T. TeNT and tetanus toxoids were diluted in binding buffer (100 mM PIPES, pH 6.4/1% BSA) to the final concentrations indicated in the text. A 100 µl of these dilutions were then added to the plate and incubated over night at 4 °C. The supernatant was discarded, and the wells were washed four times with PBS/T and once with 100 mM PIPES, pH 6.4, followed by an incubation with 100 µl of 100 mM PIPES, pH 6.4, for 45 min at 37 °C. After four washes with PBS/T, the plate was incubated for 2 h at 37 °C with tetanus rabbit antiserum BRP Batch 1 (European Directorate for the Quality of Medicines, Strasbourg, France) which had been diluted 1:4000 in PBS/0.05% Tween 20/1% BSA. The plate was washed and then incubated for 1 h at 37 °C with biotin-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany). After four washes with PBS/T, the plate was incubated for 1 h at 37 °C with streptavidin-conjugated peroxidase (Dianova). After washing five times with PBS/T, a solution containing the colorimetric substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 was added. The reaction was stopped with H_2SO_4, and the absorbance was measured at 450 nm against 620 nm as reference wavelength. All samples were measured in duplicate or triplicate, and the mean absorption values were determined. Where indicated, the values of the respective control wells (representing the non-specific binding in the absence of gangliosides) were subtracted from the measured data.

2.3. Endopeptidase assay

The assay was performed as described previously (Kegel et al., 2007; Behrensdorf-Nicol et al., 2008) with slight modifications. A MaxiSorp plate was coated with 100 µl/well of 1.5 µM rSyb2 in PBS at 37 °C for 2 h. Residual protein binding sites were blocked with PBS/5% sucrose/0.5% BSA/100 µg/ml asolectin over night at 4 °C. Then the plate was washed four times with PBS/T and once with 100 mM PIPES, pH 6.4. The toxoid samples were diluted in reducing cleavage buffer [100 mM PIPES, pH 6.4/5% sucrose/200 µg/ml asolectin/10 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP)] to the indicated final concentrations. A 100 µl of these dilutions were added to the immobilized rSyb2 and incubated for 6 h at 37 °C and 250 rpm. After the cleavage reaction, the supernatant was discarded, and the plate was washed