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Short communication

Is the test for irreversibility of tetanus toxoids still relevant?

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ABSTRACT

Tetanus vaccines for human and veterinary use are based on toxoids resulting from a formaldehyde-mediated inactivation of tetanus neurotoxin (TeNT). Due to the high toxicity of TeNT, safety tests are mandatory for each batch of these toxoids. One of the tests addresses the irreversibility of inactivation: The toxoid is stored at 37 °C for 6 weeks and then subjected to *in vivo* toxicity testing.

However, we found that TeNT solutions rapidly lose their activity at 37 °C. Accordingly, any active TeNT molecules arising in the toxoid due to reversion events may no longer be detectable after the 37 °C storage period. Furthermore, there is no evidence that a "reversion to toxicity" has ever been observed for tetanus toxoids during vaccine production. Thus, we conclude that the irreversibility test that is prescribed for human and veterinary vaccines has no relevance for vaccine safety.

1. Introduction

For many decades, tetanus toxoids resulting from a formaldehyde-mediated inactivation of tetanus neurotoxin (TeNT) have been used as vaccines to protect humans and animals from tetanus disease. Active TeNT molecules consist of two protein subunits: The heavy chain mediates the toxin uptake by neurons, and the light chain then cleaves the protein synaptobrevin and thus blocks neurotransmitter release, resulting in a spastic paralysis.

Due to the high toxicity of TeNT [1], safety tests for tetanus toxoids are mandated by the European Pharmacopoeia [2,3]. In these tests, the toxoid is injected into guinea pigs, and if none of the animals dies, the material is considered as sufficiently detoxified. For tetanus vaccines for human use, these safety tests are divided in two parts [2]: Directly after detoxification, the toxoid is tested to confirm the absence of residual toxicity as outlined above. In addition, an aliquot of the toxoid is diluted and stored at 37 °C for 6 weeks before being tested to demonstrate the irreversibility of the inactivation. As a potential "reversion to toxicity" is thought to occur only at elevated temperatures, toxoid which was stored at 5 °C serves as negative control in this test. A similar test for irreversibility after 6 weeks of storage at 37 °C and 5 °C is also required for veterinary tetanus vaccines [3].

In recent years, the relevance of these irreversibility tests has repeatedly been questioned by experts and companies in the field of vaccine manufacturing. The key questions were: Can a "reversion to toxicity" of formaldehyde-inactivated tetanus toxoids occur at all? And if a reversion does indeed occur, is the currently required test appropriate for recognizing the reverted toxoids?

With respect to the first question, a literature research showed that there is very little evidence for a "reversion to toxicity" of formaldehyde-inactivated tetanus toxoids. Formaldehyde causes a two-step reaction leading to the inter- and intra-molecular cross-linking of proteins [4]: It first reacts with a primary amino group (mainly from lysines), thereby forming a Schiff base. This step is reversible. Afterwards, the Schiff base reacts with the aromatic or heterocyclic rings of tyrosine, tryptophan or histidine, resulting in a stable and irreversible linkage of proteins via methylene bridges. Once this reaction has been completed, the toxoid is very stable against environmental factors like heat, pH changes or proteases.

It is generally assumed that a reversion to toxicity of formaldehyde-inactivated toxoids can only occur if the original detoxification process has been incomplete, so that numerous molecules have not undergone the second, irreversible reaction step [4,5]. For diphtheria toxoids, reversion events have been reported by several groups [6–8]; however, none of these reports was actually based on findings obtained with diphtheria toxoids from a validated manufacturing process. And for tetanus toxoids, it has been described that they are even less susceptible to reversion processes [4], because the TeNT molecules are larger than diphtheria toxin and contain more lysine and tyrosine residues as potential targets for the formaldehyde-induced cross-linking. In fact, we found only two short notes published in 1971 and 1993 [9,10] which describe a reversion to toxicity of tetanus toxoids after storage at 37 °C. However, both publications lack essential information about the used toxoids and the experimental

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setup; therefore their relevance for toxoids produced under controlled vaccine manufacturing conditions is highly questionable. In contrast, a large vaccine manufacturer reported in 1977 that they had never observed any reversion events with tetanus toxoids [8]. Besides, data published in 1988 [5] indicated that the "reversion" of tetanus toxoids that was reported in 1971 had probably been due to the usage of tetanus toxoids that were incompletely detoxified from the outset. So there is no conclusive evidence that reversion to toxicity has ever been observed for tetanus toxoids when using detoxification conditions that can be considered as relevant for vaccine production.

The second key question relates to the meaningfulness of the irreversibility test: Many proteins tend to rapidly lose their biological activity when stored at elevated temperatures in diluted solutions [11]. Therefore, if any active TeNT molecules should arise in the toxoids during 37 °C storage due to reversion events, these molecules may no longer be detectable in toxicity tests performed at the end of the 6-week storage period.

In order to examine whether the prescribed irreversibility test can contribute to ensuring the safety of tetanus vaccines, we tested for how long solutions containing active TeNT retain their activity during storage at 37 °C. Activity testing was performed using the BINACLE (binding and cleavage) assay, which measures the activity of TeNT *in vitro* based on its specific receptor binding and proteolytic functions [12,13].

We have presented the results obtained in this study to the European Pharmacopoeia Expert Groups for human and veterinary vaccines and sera in 2017 in order to stimulate a discussion on the relevance of the 37 °C storage test.

2. Methods

2.1. Preparation of toxin samples

Pure TeNT (Sigma-Aldrich, Taufkirchen, Germany) was diluted in water supplemented with 5 mg/ml protease-free bovine serum albumin (Serva, Heidelberg, Germany) to concentrations of 100 μg/ml, 20 μg/ml, and 4 μg/ml. Aliquots of these toxin solutions were stored at 37 °C for different periods of time and then tested in the BINACLE assay.

2.2. BINACLE assay for the activity determination of TeNT

Immediately prior to BINACLE testing, all stored TeNT samples were adjusted to a concentration of 5 ng/ml. A freshly prepared dilution series of TeNT which had not been stored at 37 °C was used to generate the standard curve. The BINACLE assays were performed as described previously [12,13]. Briefly, the samples were incubated in a microtiter plate containing immobilized ganglioside GT1b as receptor. Afterwards, bound toxin molecules were treated with a reducing agent to release the light toxin chains. The supernatant containing these light chains was then transferred to a plate containing immobilized synaptobrevin. The cleavage of this substrate protein was finally detected in an antibody-mediated reaction, which was quantified colorimetrically.

3. Results

To examine how storage affects toxin activity, TeNT solutions were tested in the BINACLE assay after incubation at 37 °C for different periods of time. Protein concentration of all samples was set to 5 ng/ml, regardless of their concentration during storage, to allow direct comparisons. Fig. 1A shows that during storage at 37 °C, the functional activity of TeNT rapidly declines: Before storage, the TeNT samples induced high absorbance signals exceeding

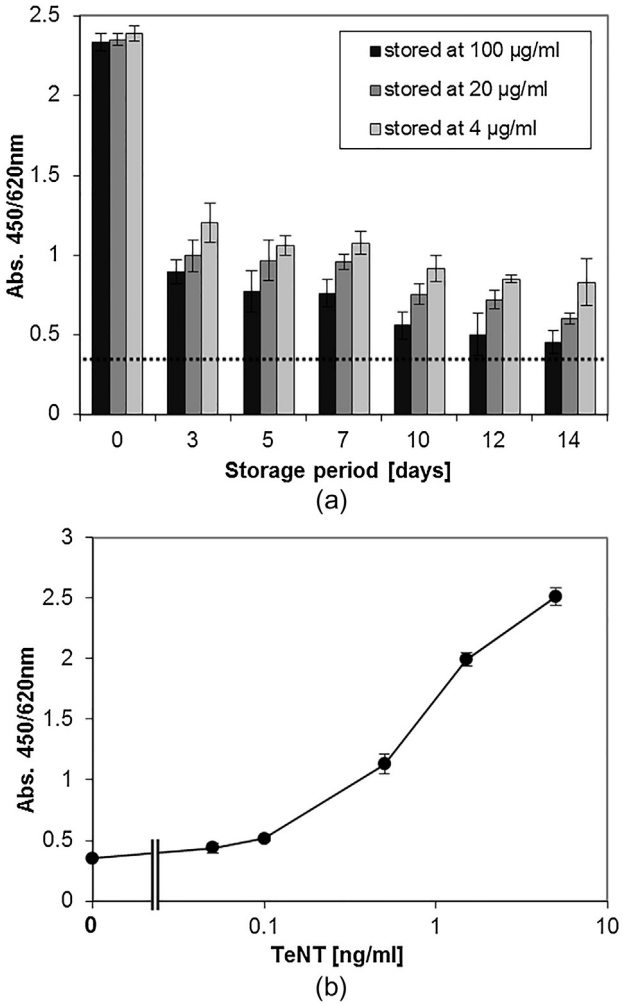

Fig. 1. Activity of TeNT samples after storage at 37 °C. (A) Aliquots of solutions containing 4, 20, or 100 μg/ml TeNT, respectively, were stored at 37 °C for periods up to 14 days. Prior to BINACLE testing, all stored solutions were diluted to 5 ng/ml. Shown are the resulting absorption signals measured at 450 nm against a reference wavelength of 620 nm. Each column represents the mean and standard deviation of 9 measured values from 3 independently stored aliquots, each measured in triplicate. The respective storage durations are indicated on the x-axis. The dotted line indicates the mean background signal which was measured in the absence of TeNT. (B) Standard curve generated in the BINACLE assay with a freshly prepared dilution series of TeNT which had not been stored at 37 °C. Each circle represents the mean absorption value and standard deviation of a triplicate measurement.

2.0, whereas already after 3 days storage at 37 °C, a considerable loss in activity was observed, resulting in signals between 0.8 and 1.2.

When comparing the stored solutions (Fig. 1A) to the standard curve (Fig. 1B), it became obvious that after three days storage at 37 °C, the activity in toxin samples tested at 5 ng/ml was similar to or even lower than in a solution containing 0.5 ng/ml of fresh toxin, corresponding to a 90% loss in activity. Storage periods between 3 and 14 days resulted in a further reduction of signal intensity (Fig. 1A), indicating a continuing decrease of toxin activity. Toxin samples that were stored in diluted form (4 μg/ml) generally showed a somewhat slower decrease in activity than concentrated toxin solutions (100 μg/ml). However, the general trend of a strong decline in activity within the first three days followed by a continuous further decrease was similar in all samples. A repeat test with slightly modified storage periods confirmed the observation that storage at 37 °C leads to rapid loss of toxin activity (data not shown).

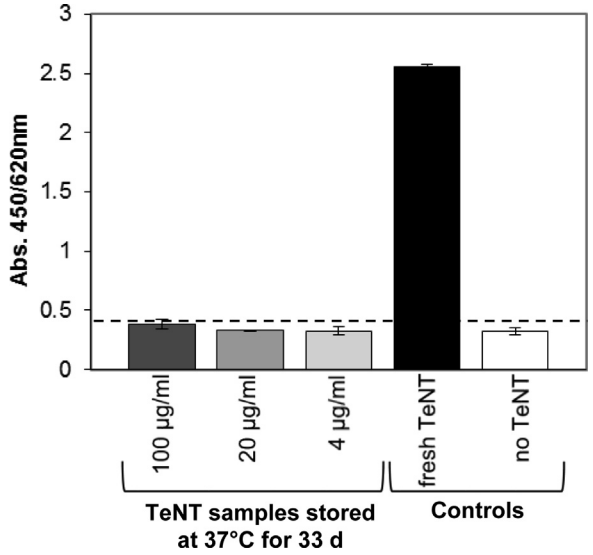

Fig. 2. BINACLE test performed with TeNT samples after storage at 37 °C for 33 days. Toxin concentrations during storage were 100, 20, or 4 µg/ml. Prior to BINACLE testing, all stored solutions were diluted to 5 ng/ml. For each storage concentration, 2 or 3 separately stored aliquots were each measured in triplicate. The negative control solution without toxin was measured in five replicates, and the fresh 5 ng/ml TeNT solution which served as positive control was measured in two replicates. Each column shows the mean value and standard deviation of the absorption values (450 versus 620 nm) that were measured for the respective type of sample or control solution. The dashed line represents the cutoff value for the detection of active toxin defined as the mean plus the 3-fold standard deviation of the absorbance values measured for the negative control solution.

To assess the effects of long-term storage, a BINACLE assay was performed with TeNT solutions that had been incubated at 37 °C for 33 days (Fig. 2). The absorption values measured for these stored samples did not exceed the mean value plus three-fold standard deviation of the negative control, which is considered as threshold for toxin detection. Also, subsequent BINACLE assays with individual toxin aliquots stored for 35 to 44 days revealed no signal above background, demonstrating that after long-term storage at 37 °C, no residual toxin activity was detectable in any of the samples irrespective of their concentration during storage.

4. Discussion

Even though the test for reversion to toxicity has been an integral part of the European Pharmacopoeia [2,3], its experimental basis has been uncertain. Here, we present an exhaustive literature overview as well as experimental data supporting the argument that the 37 °C storage test for tetanus toxoids has no relevance for vaccine safety:

The scant available literature revealed that it is highly doubtful whether a reversion of tetanus toxoids can occur at all under conditions that may be considered as relevant for vaccine production [4,5,8]. Accordingly, the necessity for reversion to toxicity testing of these products seems questionable.

Assuming "reversion to toxicity" would occur to a relevant extent, it is doubtful if the 37 °C storage test described in the European Pharmacopoeia is capable of detecting such reverted toxoids. Our experimental results demonstrate that the functional activity of TeNT rapidly decreases during storage at 37 °C: Already after 3 days, toxin solutions had lost 90% of their initial activity, and after 33 days, no residual toxin activity was detectable. Our findings that TeNT is not stable at 37 °C are in accordance with unpublished data from a study that Dorothea Sesardic performed several years ago [personal communication, December 2018]: She showed in a mouse paralysis assay as well as in an endopeptidase assay that TeNT diluted in toxoid solutions had lost all of its activity after six weeks of storage at 37 °C. Accordingly, any active TeNT molecules which might arise in the toxoids during storage at 37 °C due to a reversal of formaldehyde-induced crosslinks can be expected to quickly lose their toxicity again in the remaining storage period. Thus, such molecules can no longer induce toxic reactions at the time when the toxoid is tested after the prescribed 6 week storage period.

The assumption that the irreversibility test does not contribute to the safety of tetanus vaccines is further substantiated by the experiences of relevant producers of tetanus vaccines who stated that in their companies, the test for irreversibility of tetanus toxoids after storage at 37 °C had never contributed to the detection of any toxoid batches with insufficient safety profiles [8; Shahjahan Shaid, GSK, personal communication, January 2019; Imke Kross, Intervet International BV, personal communication, January 2019].

Considering the lack of evidence for reversion events during the production of tetanus vaccines, the necessity for the irreversibility test as a safety test for tetanus toxoids is highly questionable. Particularly with regard to animal welfare, we think that the requirement to perform this test should no longer be maintained. For this reason, we presented our experimental data concerning the stability of TeNT during 37 °C storage to the Pharmacopoeia Expert Groups for human and veterinary vaccines and sera in autumn 2017. We propose that based on our results, and with respect to the fact that no indications were found which substantiate a beneficial effect on vaccine safety, the toxoid storage tests prescribed for human and veterinary tetanus vaccines should be deleted from the Pharmacopoeia monographs 0452 and 0697.

The deletion of the test for irreversibility of tetanus toxoids due to its lack of relevance would be highly welcome: It would reduce the animal experiments required for batch testing of tetanus vaccines in accordance with the European Directive 2010/63/EU [14], and in addition it would save time and costs during manufacturing.

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Conflict of interest statement

Declarations of interest: none.

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