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Physicochemical and immunochemical assays for monitoring consistent production of tetanus toxoid

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ABSTRACT

The detoxification of tetanus toxin by formaldehyde is a crucial step in the production of tetanus toxoid. The inactivation results in chemically modified proteins and it determines largely the ultimate efficacy and safety of the vaccine. Currently, the quality of tetanus toxoid lots is evaluated in potency and safety tests performed in animals. As a possible alternative, this article describes a panel of *in vitro* methods, which provides detailed information about the quality of tetanus toxoid. Ten experimental lots of tetanus toxoid were prepared using increasing concentrations of formaldehyde and glycine to obtain tetanus toxoids having differences in antigenicity, immunogenicity, residual toxicity and protein structure. The structural properties of each individual toxoid were determined using immunochemical and physicochemical methods, including biosensor analysis, ELISA, circular dichroism, TNBS assay, differential scanning calorimetry, fluorescence and SDS-PAGE. The quality of a tetanus toxoid lot can be assessed by these set of analytical techniques. Based on antigenicity, immunogenicity and residual toxicity data, criteria are formulated that tetanus toxoids lot have to meet in order to have a high quality. The *in vitro* methods are a valuable selection of techniques for monitoring consistency of production of tetanus toxoid, especially for the detoxification process of tetanus toxin.

1. Introduction

Tetanus toxoid is chemically detoxified tetanus toxin, which is used for the production of tetanus toxoid-containing vaccines and as a constituent of polysaccharide-conjugate vaccines, such as haemophilus type B, meningococcal group C and pneumococcal polysaccharide vaccines. The manufacturing of tetanus vaccines comprises the cultivation of *Clostridium tetani*, harvest and inactivation of tetanus toxin, the purification of tetanus toxoid, and finally the adsorption of tetanus toxoid to a colloidal aluminium salt. The production is very similar to that of the diphtheria vaccine. In 1925 the inactivation procedure using formaldehyde was described for the first time by G. Ramon and P. Descombey [1]. This chemical treatment still is used today. It is a critical step, which determines significantly the quality of the final vaccine. The reaction conditions used for the detoxification can be slightly different for each producer, such as formaldehyde concentration, reaction time, reaction temperature, pH and composition of the detoxification matrix [2]. Although the chemistry of formaldehyde reacting with proteins is well-known [3–5], its effect on the product quality of tetanus vaccines remains unclear.

Quality control of the classical tetanus toxoid vaccines still relies on extensive testing in animals. Prior to lot release, the potency and safety of every final vaccine lot has to be determined in an *in vivo* test. Each vaccine lot is regarded as a unique product. In general, vaccines are complicated and heterogeneous products, which contain active components, adjuvants and excipients. Due to this complexity, substantial differences in the quality of vaccine lots might be observed. Therefore, regulatory authorities require that quality control tests are performed on each vaccine lot [6]. Furthermore, incidents with vaccines and biologicals led to rigorous quality control of vaccines [7,8]. Nevertheless, significant improvements have been made in current vaccine production and the required controls. The optimisation and standardisation of the production process (*e.g.* according to Good Manufacturing Practices, GMP) and intensive in-process controls have resulted in vaccine lots of consistent, high quality [6,9]. Once overall consistency of production is demonstrated, regulatory authorities should

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reconsider the omission of a potency testing in animals. In principle, analytical methods can replace traditional testing of toxoid vaccines in animals by monitoring the crucial characteristics of intermediate and final products. Currently, ELISA and enzymatic assays are described as tools for quality control of tetanus vaccines [10–12]. However, the number of analytical tests for tetanus vaccines is too limited to determine consistency of production and quality of the final lot. Additional assays are needed that can verify that a newly produced lot has the same characteristics, in terms of physicochemical properties, compared to a lot which has proven to be efficacious and safe.

The question rose: can physicochemical and immunochemical assays be used to distinguish between tetanus toxoids with an inferior or a high quality? For that reason, ten experimental tetanus toxoids were prepared by using increasing concentrations of formaldehyde en glycine. These tetanus toxoids showed differences in antigenicity, immunogenicity and residual toxicity. A set of physicochemical and immunochemical assays were developed to monitor the structural properties of these tetanus toxoids. Ultimately, these *in vitro* methods can be valuable test panel for the quality control of tetanus toxoids.

2. Animals, materials and methods

2.1. Immunochemicals

The monoclonal antibody 14F5 was purchased from Abnova (Taiwan). Monoclonal antibodies 18.134112, QC07-01, QC07-04, and TF94/1-2-20 were homemade antibodies.

2.2. Cultivation and purification of tetanus toxin

A culture of *C. tetani E88* (Massachusetts strain) was grown in a bioreactor (Applikon, NL) containing 15 L of Mueller & Miller medium [13]. Tetanus toxin-containing culture fluid was diafiltrated using 10 mM sodium phosphate, pH 7.0 and concentrated ten times using a tangential flow filtration system (Millipore) equipped with a Pellicon XL 50 filter (MWCO: 30 kDa). The toxin-containing fluid was diluted seven times in 20 mM piperazine dihydrochloride, pH 5.5. The toxin was purified by ion exchange chromatography using a column (XK 26/20; GE Healthcare) packed with Q Sepharose Fast Flow (GE Healthcare). The unbound fraction was collected which contained the tetanus toxin. The collected, toxin-containing fluid was 20 times concentrated prior to size exclusion chromatography. The separation was performed on a column (AxiChrom 50/500) packed with Superdex 200 prep grade (GE healthcare) using PBS (1.5 mM KH_2PO_4, 2.7 mM Na_2HPO_4-$7H_2O$ and 155 mM NaCl; Gibco) as an eluent. Fractions containing the tetanus toxin were pooled and concentrated to a final protein concentration of about 0.4 mg/ml.

2.3. Experimental tetanus toxoids

For the preparation of ten experimental tetanus toxoids, equimolar concentrations of glycine and formaldehyde solutions were added to 0.4 mg/ml tetanus toxin in solution (Table 1). PBS was added to the samples to obtain a final protein concentration of 0.25 mg/ml, and formaldehyde and glycine concentrations of 1, 2, 4, 8, 16, 32, 48, 64, 128, and 150 mM, respectively. The mixtures were incubated for 7 weeks at 35 °C. The toxoids were stored at 4 °C prior to analysis or adsorption to aluminium phosphate.

Table 1
Tetanus toxin and experimental tetanus toxoids.

Name	Formaldehyde and glycine (mM)[a]
TTn	–
TTd1	1
TTd2	2
TTd3	4
TTd4	8
TTd5	16
TTd6	32
TTd7	48
TTd8	64
TTd9	128
TTd10	150

[a] Tetanus toxoids were prepared by adding indicated concentrations of formaldehyde and glycine to tetanus toxin. The final protein concentration in each sample was 0.25 mg/ml.

2.4. ELISA

The antigen concentrations present in the toxoid samples were determined by a sandwich ELISA. ELISA plates (Greiner) were coated overnight at ambient temperature with 333 × diluted horse anti-tetanus toxoid serum (NVI) in 40 mM sodium carbonate buffer, pH 9.6. (The antiserum was obtained from a horse that is immunised with tetanus toxoid and received a booster with tetanus toxin several times.) After the incubation, plates were washed twice with 0.03% (v/v) Tween 80 in water. Then, plates were incubated for 2 h at 37 °C with twofold serial dilutions of the toxoid samples and a reference DT79/1 (NVI), diluted in PBS, pH7.2 (Gibco) with 0.05% (v/v) Tween 80. Plates were washed and incubated for 1.5 h at 37 °C with 4000 × diluted horse anti-tetanus toxoid conjugate (NVI) in PBS with 0.05% (v/v) Tween 80 and 0.05% (w/v) protifar (Nutricia). After washing, peroxidase substrate (25 mM TMB and 0.01% H_2O_2 in 0.11 M sodium acetate buffer, pH 5.5.) was added, plates were incubated at ambient temperature and the reaction terminated after 10 min by adding 2 M H_2SO_4. Finally, the absorbance was recorded at 450 nm with a plate reader (Biokinetics reader EL312e, Bio-tec instruments). A reference (NVI) containing 150 Lf/ml tetanus toxoid was used to determine the antigen concentrations (Lf/ml) present in the toxoid samples. The antigenicity of tetanus toxoids was plotted against the formaldehyde concentration (mM) used for detoxification. The experiment was performed in quintuplet. Significant differences in the antigenicity of the toxoids TTd1-TTd10 were determined using ANOVA. Curve fitting was performed according the function of the dose response distribution ($y = a+b/(1+(x/c)^d)$) using TableCurve 2D (Systat Software GmbH, Germany).

2.5. Specific toxicity

Residual toxicity present in the experimental toxoid samples was determined in an *in vivo* study, because a validated *in vitro* assay is not yet available. Three mice (NIH, female, weight 17–21 g) were injected subcutaneously in the groin with 250 μl tetanus toxin or toxoid. The mice were observed for 21 days to check for signs caused by tetanus toxin. Mice that showed signs of typical muscular spasms of the hind leg were immediately euthanized.

2.6. Adsorption onto aluminium phosphate

Tetanus toxoids were dialysed (MWCO: 10 kDa) using PBS and subsequently diluted in an aluminium phosphate mixture (1.5 mg/ml $AlPO_4$ in 0.15 M saline) to a final concentration of 0.4 Lf/ml (1–6 μg/ml). The samples were mixed for 24 h at 4 °C. The adsorption degree of tetanus toxoid onto aluminium phosphate was determined by a sandwich ELISA. The adsorption degree for all tetanus toxoids was 100% (data not shown).

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2.7. Immunogenicity

The immunogenicity of the experimental tetanus vaccines was determined in mice. Eight mice (NIH, female, weight 10–14 g) were injected subcutaneously in the groin with 250 μl of a vaccine (0.1 Lf tetanus toxoid). A reference vaccine DTa 93/1 with a potency of 27.2 IU/Lf was used as a reference. After 35 days, the animals were bled and the blood was collected individually. The blood was incubated for 2 h at 37 °C and subsequently for 2 h at 4 °C. The samples were centrifuged for 20 min at 800 g. The supernatants were transferred into new tubes, and centrifuged once again to obtain cell-free sera. Then, sera were again transferred into new tubes and complement was inactivated by heating at 56 °C for 45 min and stored at −20 °C prior to analysis. For the serological estimation of the immunogenicity, the toxin binding inhibition test (ToBI) was performed as described previously [14]. Significant differences in the immunogenicity of the toxoids TTd2-TTd10 were determined by using ANOVA. The immunogenicity (IU/Lf) of tetanus toxoids was plotted against the formaldehyde concentration (mM) used for detoxification. Curve fitting was performed according the function of the log normal distribution ($y = a+b\cdot\exp(-0.5(\ln(x/c)/d)^2)$) using TableCurve 2D (Systat Software GmbH, Germany).

2.8. SDS-PAGE

SDS-PAGE was performed under native, non-reducing and reducing conditions. For native SDS-PAGE, 3–7 μg protein was diluted in non-reducing sample buffer (60 mM Tris, 70 mM SDS, 0.1 mM tetrabromophenol blue and 35% glycerol diluted in water) to a volume of 20 μl. For non-reducing conditions, 3–7 μg protein was diluted in non-reducing sample buffer to a volume of 20 μl and boiled at 100 °C for 15 min. For reduction of the disulphide bridges, 2 μg of the protein was diluted in reducing sample buffer (60 mM Tris, 70 mM SDS, 0.1 M dithiothreitol, 0.1 mM tetrabromophenol blue and 35% glycerol diluted in water) to a volume of 20 μl and boiled at 100 °C for 15 min. The total sample volumes of 20 μl were loaded onto 8% SDS-PAGE gels (Pierce) and electrophoretically separated at 100 V. SDS-PAGE Molecular weight (broad range; Bio-Rad) were used as a reference. Protein bands were visualised by using Imperial Protein Stain (Pierce). The gels were scanned and the intensity of protein bands was quantified using ImageJ 1.46r software (NIH, USA).

2.9. TNBS assay

The reaction of formaldehyde with tetanus toxin results in reduction of the number of primary amino groups. The number of primary amino groups in the experimental tetanus toxoids was determined by using a colorimetric assay [20]. Prior to the analysis, toxoid samples were dialysed against PBS (Gibco) to remove unreacted glycine. After the dialysis, the protein concentration of each sample was determined by the BCA protein assay (Pierce) and the concentration of amino groups by using 2,4,6-trinitrobenzenesulphonic acid (TNBS) [20].

2.10. Fluorescence

The guanidine-HCl-induced denaturation of tetanus toxin and the experimental tetanus toxoids was studied by fluorescence spectroscopy. Toxoid samples (25 μg/ml) were incubated for 2 h with guanidine-HCl concentrations ranging from 0 to 8 M in steps of 0.25 M. The spectra of the toxoids were recorded at 25 °C with a Perkin Elmer LS55B fluorescence spectrometer. The excitation wavelength was 295 nm (bandwidth of 2.5 nm) and the emission wavelength was measured between 300 and 450 nm (bandwidth of 3.5 nm). For each sample, the emission maximum was determined after calculating the average of five consecutive scans (corrected for background). The guanidine-HCl concentration needed to denature 50% of the molecules of a particular tetanus toxoid was plotted against the formaldehyde concentration (mM) used for detoxification. Curve fitting was performed according the function of the log normal distribution ($y = a+b\cdot\exp(-0.5(\ln(x/c)/d)^2)$) using TableCurve 2D (Systat Software GmbH, Germany).

2.11. DSC

Tetanus toxoid was diluted in PBS (Gibco) to a concentration of 0.20–0.25 mg/ml. The sample cell of a micro differential scanning calorimeter (MicroCal VP-Capillary DSC System; GE Healthcare) was loaded with 300 μl toxoid and the reference cell was filled with PBS containing equimolar concentrations of glycine. The cells were kept at 20 °C for 10 min before starting the measurement. Thermograms were collected between 20 and 110 °C with a scan rate of 3 °C/min. From the thermograms, the temperature of denaturation (Td) was calculated using Origin 7.0 software (OriginLab Corporation, USA). The denaturation temperatures were plotted against the formaldehyde concentration (mM), which used for detoxification of tetanus toxoids. Curve fitting was performed according the function of the log normal distribution ($y = a+b\cdot\exp(-0.5(\ln(x/c)/d)^2)$) using TableCurve 2D (Systat Software GmbH, Germany).

2.12. Circular dichroism

Circular dichroism (CD) spectra were recorded at ambient temperature with a Chirascan CD Spectrometer (Applied Photophysics, UK) equipped with a 150 W Xenon AVC lamp. Far-UV CD spectra were taken from 260 to 190 nm in steps of 0.5 nm and a scan rate of 5 s-1. The bandwidth of the monochromator was set on 1 nm. The concentration of the experimental tetanus toxoids added to the cuvettes (10 mm) was 5 μg/ml. The Far-UV CD spectra were smoothed (moving average of 13). The measured CD signals were converted to molar ellipticity ($\Delta\varepsilon$), based on a mean residual weight of 115. CDNN software (Applied Photophysics, UK) was used to calculate the content of α-helix, β-sheet, β-turn and random coil.

2.13. Biosensor analysis

Biosensor analyses were performed on a surface plasmon resonance system (Biacore T100, GE healthcare) to measure the binding of particular monoclonal antibodies to the tetanus toxoids. Therefore, Fc-specific antibodies were coupled to a CM5 chip (Biacore) which results in an increase of 6000 resonance units (RU). Then, a tetanus toxin-specific monoclonal antibody was captured by the Fc-specific antibodies using an injection time of 2 min and a flow of 10 μl/min (The binding of monoclonal antibodies was between 150 and 300 RU, dependant on which antibody was used.) After an equilibration time of 7 min, 25 μl of an individual tetanus toxoid was injected with a flow of 5 μl/min. The association and the dissociation were measured for a period of 5 and 10 min, respectively. Background signals were determined in a reference flow cell and subtracted using Biacore T100 Evaluation Software 2.0.3 (Biacore).

3. Results and discussion

3.1. Quality of experimental tetanus toxoids

Ten experimental tetanus toxoids were prepared using increasing concentrations of formaldehyde and glycine (Table 1). The antigenicity, residual toxicity and immunogenicity of each individual

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toxoid were investigated. At first, the antigenicity was determined in the ELISA using polyclonal antiserum, and compared to the protein concentration (Fig. 1A). The test revealed a significant reduction (ANOVA: $p = 0.0001$) in the antigenicity if tetanus toxin was treated with formaldehyde and glycine concentrations above 16 mM. The ability of polyclonal antiserum to bind to the toxoids is decreased, probably by the disappearance of particular epitopes because of chemical modifications in tetanus toxoid molecules.

The minimal concentration of formaldehyde and glycine needed for complete detoxification of tetanus toxin was determined with the *in vivo* toxicity test. Mice revealed no clinical signs after the injection of tetanus toxoid when it was prepared with formaldehyde and glycine concentrations equal to or higher than 2 mM. This observation corresponds with results described in the literature, although a different detoxification matrix was used [15]. The formaldehyde concentration needed to detoxify tetanus toxin completely was much lower than needed for, *e.g.* diphtheria toxin (16 mM formaldehyde). Both tetanus and diphtheria toxin belong to the group of AB toxins because of their structural composition [16]. Formaldehyde inactivates tetanus toxin most likely by stopping of at least one of its physiological activities, such as receptor binding, retrograde transport, translocation or enzyme activity [12]. The B-fragment of tetanus toxin comprises the binding and translocation domains which delivers the enzymatically active A-fragment into the cytosol, inhibiting the release of neurotransmitters [17].

In addition, the immunogenicity (IU/Lf) was determined for the tetanus toxoids by use of the ToBI procedure [18,19]. The immunogenicity of TTn and TTd1 was not tested in mice because of residual toxicity. The results of the immunogenicity tests are given in Fig. 1B. Based on statistical evaluation, toxoids can be divided in two groups: (I) TTd2–TTd5 and (II) TTd6–TTd10, The immunogenicity was comparable within groups I and II (ANOVA; $p = 0.33$ and $p = 0.85$, respectively). A significant increase (ANOVA, $p = 0.0001$) in the immunogenicity was observed for group II if compared to group I. The phenomenon of an improved immunogenicity by formaldehyde treatment was also observed for diphtheria toxoid [2]. One of the explanations is the increased stability of the toxoid by the formation of formaldehyde-induced cross-links. Presumably, the internal cross-links in the toxin protect against rapid proteolytic degradation after immunisation and improving the immunogenicity [20].

The tetanus toxoid vaccines were prepared with equal antigen content (0.4 Lf/ml) instead of equal protein content, as is done with routine products. In addition, the dynamic range of the animal test is small (0.05 Lf/ml–0.75 Lf/ml), increasing the risk of dosing outside the limits of quantification. The immunogenicity based on protein concentration (IU/μg) could also be recalculated for toxoids TTd2–TTd10 (data not shown). This was possible because for each toxoid the antigenicity and protein concentration were determined accurately (Fig. 1A). The calculated immunogenicity standardised on protein (IU/μg) did not increase. Immunogenicity was about 5.6 IU/μg protein for TTd2 and 4.0 IU/μg protein for TTd10. In conclusion, the formaldehyde treatment has a significant effect on toxicity, antigenicity and immunogenicity standardised on antigen concentration.

3.2. Structural changes in tetanus toxoid visualised by SDS-PAGE

Tetanus toxin (150 kDa) is composed of two fragments of 50 kDa (A-fragment) and 100 kDa (B-fragment) which are connected via a disulphide bridge [17]. After reduction of the disulphide bridges, tetanus toxin (TTn) and the ten experimental toxoids (TTd1–TTd10) were separated in an acrylamide gel. Two protein bands with expected masses of the A and B fragments from TTn were visible on the gel (Fig. 2A, lane 1). A protein band appeared at 150 kDa after the electrophoresis when tetanus toxoids were treated with

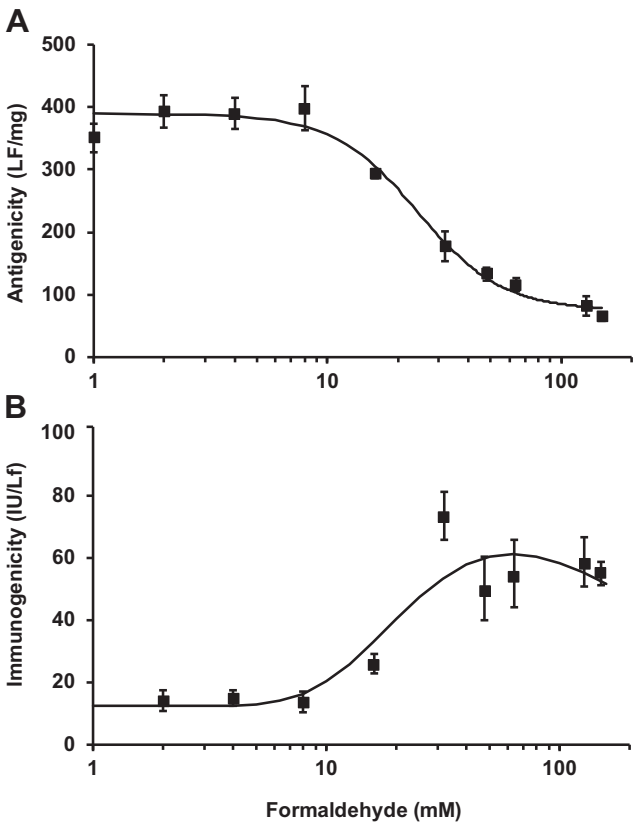

Fig. 1. Specific antigenicity (A) of TTd1–TTd10 and immunogenicity (B) of TTd2–TTd10. The antigenicity (Lf/mg protein) was measured in an ELISA by using horse anti-tetanus toxoid serum. The antigenicity of tetanus toxin was 400 Lf ± 22 Lf/mg. The immunogenicity of TTd2–TTd10 was determined in mice using vaccines with a constant antigenicity level (0.4 Lf/ml). In (A) and (B), the correlation coefficients (R^2) of the fitted lines were 0.98 and 0.85, respectively.

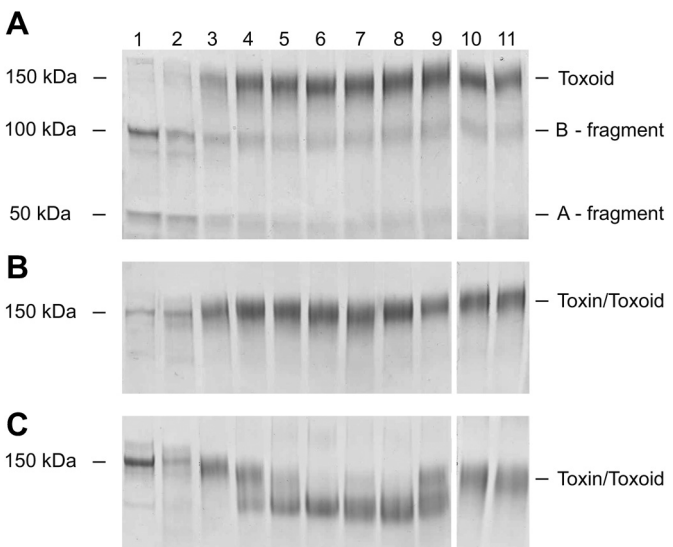

Fig. 2. SDS-PAGE of tetanus toxin (lane 1) and toxoids DTd1–DTd10 (lane 2–11, respectively). The electrophoresis was performed on (A) under reducing conditions, (B) non-reducing conditions and (C) native conditions (details given in M&M). Tetanus toxoids were prepared with increasing concentrations of formaldehyde and glycine (Table 1).

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formaldehyde and glycine concentrations of 2 mM or higher (Fig. 2A, lane 3–11). In addition, the intensities of the 50 kDa and 100 kDa protein bands were strongly decreased for TTd2–TTd10. Because of the chemical treatment, the A and B fragments were already cross-linked in tetanus toxoid at rather low concentrations of formaldehyde (2 mM). More than 63% of the A and B fragments were cross-linked in TTd2.

The percentage of cross-linked A and B fragments was 70–80% for TTd3–TTd10. In addition, tetanus toxin and toxoids were separated on an acrylamide gel after boiling the samples for 15 min without using reducing conditions. The protein bands were visible at 150 kDa because of the intact disulphide bridges (Fig. 2B). However, the protein bands became more diffuse upon formaldehyde treatment. The formation of diffuse protein bands is most likely a result of variable numbers of intramolecular cross-links and diverse reaction products attached to the tetanus toxin molecules [2,4].

Finally, the toxoids were separated on an acrylamide gel without boiling the samples and reduction of the sulphide bridges (Fig. 2C). The protein bands of the individual toxoids (TTd3–TTd8) treated with 4–64 mM formaldehyde and glycine appeared at a lower mass (~100 kDa). Presumably, the formed cross-links may give the toxoids a more compact structure which resulted in a faster migration through the gel [2,21]. The toxoids TTd9 and TTd10 demonstrated a slower electrophoresis in the acrylamide gel. Probably these toxoids contain less intramolecular cross-links and more attached glycine molecules by the formaldehyde treatment.

3.3. Reduction of primary amino groups

During the detoxification process, formaldehyde reacts primarily with the lysine residues in tetanus toxin to form intramolecular cross-links [4]. Tetanus toxin contains 108 primary amino groups [22]. The formaldehyde reaction resulted in a reduced number of available primary amino groups. The reduction was determined for each individual toxoid (Fig. 3). The TNBS assay revealed vast decrease of primary amino groups when using a low concentration of formaldehyde (1 mM). Treatment with higher formaldehyde concentrations showed an additional reduction in the number of primary amino groups, except for TTd4. In contrast to tetanus toxoid, diphtheria toxoid demonstrated a gradual decrease in primary amino groups upon treatment with increasing formaldehyde concentrations [2]. It is difficult to explain the difference between tetanus and diphtheria toxoid. Probably, the formation of intramolecular cross-links is more effective in tetanus toxin at low formaldehyde concentrations than in diphtheria toxin.

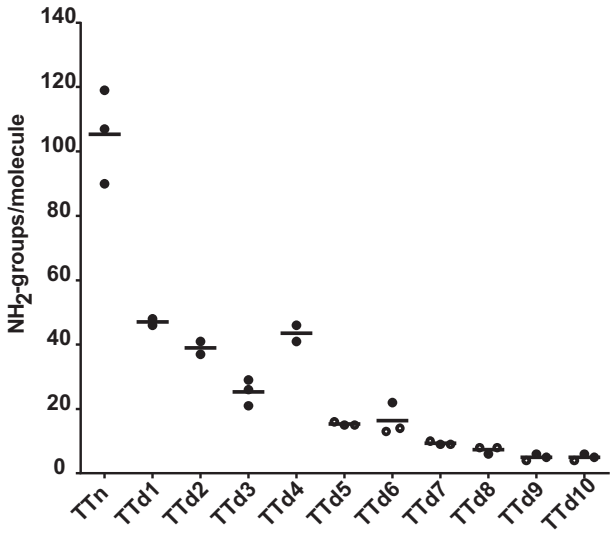

Fig. 3. The number of primary amino groups in tetanus toxin and experimental toxoids were determined by the TNBS assay (values and mean; $n = 3$, except for TTd1, TTd2 and TTd4 ($n = 2$)).

3.4. Structural stability of tetanus toxoids

The stability of the protein conformation is improved by the formation of intramolecular cross-links caused by formaldehyde treatment [2,21,23]. The conformational stability was studied for tetanus toxoids (TTd1-TTd10) to reveal the effect of different formaldehyde and glycine concentrations during detoxification. Denaturation of the toxoids by guanidine-HCl was monitored by fluorescence spectroscopy. The 13 tryptophan residues in tetanus toxin gave a fluorescence emission maximum around 337 nm under physiological conditions. The emission maximum shifted to 354 nm upon denaturation by guanidine-HCl. The guanidine-HCl concentration needed to achieve 50% shift of the emission maximum was determined (Fig. 4A). The results showed that tetanus toxoids became more resistant to denaturation when increasing formaldehyde concentration was applied. The guanidine-HCl concentrations needed for denaturation was slightly reduced for toxoids TTd9 and TTd10, which were treated with formaldehyde concentrations higher than 128 mM.

The temperature of denaturation was determined for tetanus toxin and a number of experimental toxoids (Fig. 4B). DSC measurements showed that tetanus toxin had a denaturation temperature of 63 °C. The denaturation temperature of the tetanus toxoid was considerably increased after the treatment with formaldehyde. The highest denaturation temperature (95 °C) was found for tetanus toxoid detoxified with 32 mM formaldehyde. The denaturation temperature was slightly decreased for toxoids TTd9 and

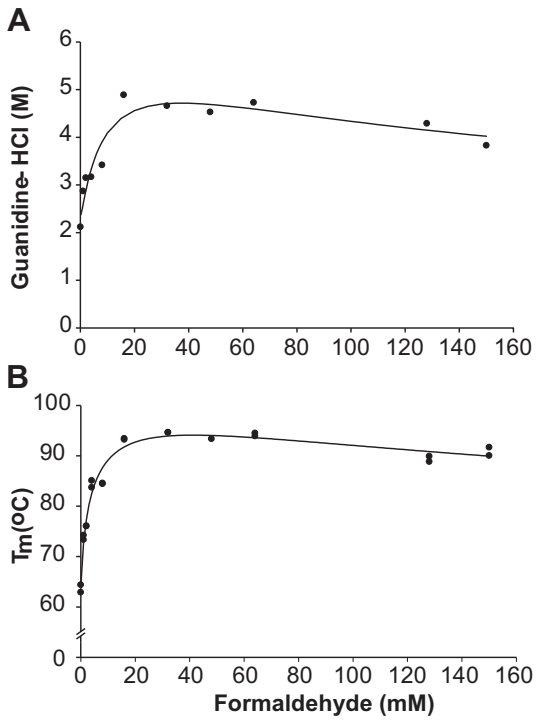

Fig. 4. The conformational stability of tetanus toxoids is determined by (A) using increasing concentrations of guanidine-HCl and (B) by heating. The denaturation by guanidine-HCl was monitored by fluorescence spectroscopy. The temperature of denaturation has been determined by differential scanning calorimetry. In Figure 4.A and 4.B, the correlation coefficients (R^2) of the fitted lines were 0.85 and 0.98 respectively.

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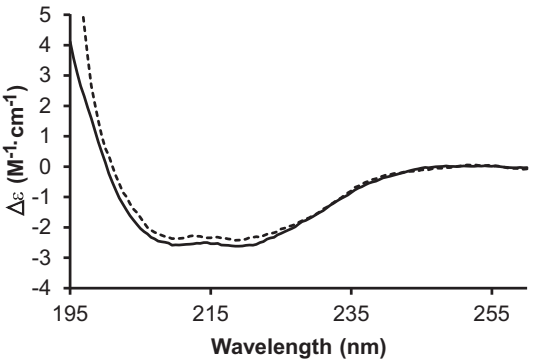

Fig. 5. Far-UV CD spectra were recorded from tetanus toxin (TTn) and the experimental toxoid (DTd9).

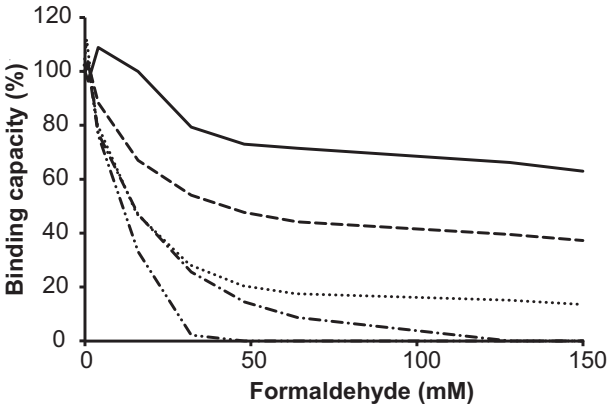

Fig. 6. The relative binding capacity of experimental tetanus toxoids to five monoclonal antibodies, i.e. 14F5 (solid), TF94/1-2-20 (dash), 18-135453 (dot), QC07-04 (dash dot) and QC07-01 (dash dot dot). The tetanus toxoids were treated with increasing concentrations of formaldehyde. The binding intensity of tetanus toxin to a particular monoclonal antibody was set to 100 percent.

TTd10. A strong linear correlation ($R^2 = 0.89$) was determined between denaturation of toxoids with guanidine and heat (data not shown). Furthermore, the denaturing characteristics were in accordance with the results found by SDS-PAGE analysis using the native conditions. The most stable toxoids (TTd3 - TTd8) migrate more rapidly through the acrylamide gel (Fig. 2C), probably because of their compact structure caused by the high number of intramolecular cross-links.

3.5. Secondary structure of tetanus toxoids

The effect of formaldehyde on the secondary structure of tetanus toxin was monitored using circular dichroism. Far-UV CD spectra of tetanus toxin and the tetanus toxoids were essentially the same. Two representative spectra are given in Fig. 5. The spectra indicate that the secondary structure of tetanus toxin was not disturbed by the treatment with formaldehyde. It seems that formaldehyde treatment in general does not perturb the secondary structure of proteins, which was earlier described for diphtheria toxin and toxoid [2]. Based on the deconvolution of CD spectra, the content of structural elements could be calculated (Table 2). Tetanus toxin has about 28% α-helices, 23% β-sheets, 18% β-turns and 34% random coils. The crystal structure of tetanus toxin has not yet been published. Only the crystal structures of both the catalytic domain and the receptor domain are described in the literature [24–26]. It remains to be seen what the actual secondary structure is based on the crystal structure.

3.6. Epitopes modified in tetanus toxoid

Formaldehyde treatment affects the antigenicity of tetanus toxin, as was confirmed by the ELISA analysis using polyclonal antiserum (Fig. 1A). In addition, five monoclonal antibodies recognising different epitopes were used to monitor the binding of each individual toxoid (Fig. 6). All monoclonal antibodies showed a reduction in binding to toxoids TTd6–TTd10, which were treated with formaldehyde concentrations above 32 mM formaldehyde.

Table 2
Structural composition of tetanus toxin and tetanus toxoids based on CD spectra.

Content	Tetanus toxin	Tetanus toxoid
α-helix	28 ± 1[a]	29 ± 2
β-sheet	23 ± 1	21 ± 1
β-turn	18 ± 0	18 ± 0
Random coil	34 ± 1	35 ± 1

[a] Mean values ± SD ($n = 5$).

The epitopes of monoclonal antibody QC07-01 and QC07-04 were completely disappeared after treating tetanus toxin with formaldehyde above 48 mM and 128 mM, respectively. In addition, a decrease in binding was detected for monoclonal antibodies 14F5, TF94/1-2-20 and 18-135453 (maximum decline of 37%, 63% and 86%, respectively). In conclusion, all epitopes recognised by the five monoclonal antibodies were affected to a greater or lesser extent. In the case of diphtheria toxoid, also some particular epitopes were destroyed by formaldehyde treatment [2]. However, other epitopes remained intact. The disappearance of the epitopes is caused by the chemical modifications of amino acid side chains, instead of conformational changes [2]. Indeed, the secondary structure was not affected by the formaldehyde treatment as was shown by circular dichroism.

4. Conclusions

The current concept of the quality assurance of vaccines is based on the consistency of production [27]. For tetanus toxoid vaccines, important release criteria are the antigen content, purity, residual toxicity, degree of adsorption and the potency [28,29]. However, none of these quality control tests focuses directly on the structural integrity of tetanus toxoid lots during production. Detoxification by formaldehyde is one of the most critical steps in the production of toxoid vaccines. Other important stages are purification and adsorption to an aluminium-containing adjuvant. Detoxification affects the structure of antigens considerably as was shown for both diphtheria and *Clostridium difficle* toxoids [2,30]. Little was known about the effect of formaldehyde on the structure of tetanus toxoids.

This article describes a panel of analytical tests that can monitor the structural changes in tetanus toxoid that occur upon formaldehyde treatment. The assays revealed that formaldehyde modifies significantly the primary structure of tetanus toxin: covalent cross-links between the A and B fragments of tetanus toxin were formed, and the number of primary amino groups was significantly reduced. In addition, several epitopes of tetanus toxin were affected by formaldehyde. Antigenic changes were not reflected in correlating changes in immunogenicity, indicating that immune assays cannot predict immunogenicity, but can be used to demonstrate consistent product manufacturing. On the other hand, the secondary structure was hardly changed and the structural stability was considerably improved.

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The experimental toxoids could be divided in two groups based on their quality features:

(i) TTd1–TTd5 showed residual toxicity or a low immunogenicity (IU/Lf)) and (ii) TTd6–TTd10 had a high immunogenicity. The test panel used can distinguish tetanus toxoids of high quality using the following criteria:
(i) the percentage of A and B fragments that were cross-linked was more than 70%, (Fig. 2A)
(ii) the number of amino groups still present in toxoid molecules is between 9 and 15 (Fig. 3)
(iii) the concentration of guanidine-HCl needed to denature half of the molecules is above 4.4 M (Fig. 4A)
(iv) the denaturation temperature is above 90 °C (Fig. 4B)
(v) the secondary structure of tetanus toxoid is not changed during detoxification (Fig. 5), and
(vi) the binding of tetanus toxoid to monoclonal antibody 14F5 is still above 75%, whereas to QC07-01 binding is less than 33% if compared to tetanus toxin (Fig. 6).

In conclusion, the combination of analytical techniques revealed structural properties of the tetanus toxoids, which are indicative for the quality of the toxoids. The test panel can discriminate between toxoids with residual toxicity or low immunogenicity (IU/Lf) and toxoids with high immunogenicity. The assays are applicable to demonstrate consistent detoxification of tetanus toxin. The validity of the tests has to be determined in extensive validation studies using routinely produced tetanus toxoids. Once consistency has been proven in all stages of production, i.e. cultivation, detoxification, purification and adsorption, the animal experiments to determine the potency of tetanus vaccines can be reduced or even omitted.

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