Structural Characterisation of Diphtheria Toxoid

Structural Characterisation of Diphtheria Toxoid

Structurele Karakterisering van Difterietoxoïd
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

Introduction

The present quality control of classical inactivated vaccines is predominantly based on potency and safety tests in laboratory animals. These tests have to be performed during the production process of a vaccine and afterwards for the release of final lots. Although substantial progress has been achieved in the reduction of animal use, the number of animal tests for the quality control is still tremendous. For example, a potency test for final-lot release of diphtheria toxoid vaccines requires at least 100 guinea pigs. The number of animals could be reduced drastically if new *in vitro* characterisation methods would be available for quality control of vaccines. Currently, no biochemical assays are applied to monitor consistency in quality of classical inactivated vaccines, such as diphtheria and tetanus vaccines.

Many countries included diphtheria vaccines in their national immunisation programs for children. At present, several vaccines are available containing diphtheria toxoid formulated with other antigens, such as tetanus toxoid, inactivated polioviruses, and pertussis bacteria (whole-cell) or pertussis antigens (acellular). The frequent usage of diphtheria toxoid-containing vaccines makes the development of *in vitro* assays for quality control of these vaccines very attractive. Moreover, these tests are often less time-consuming, cheaper and more precise than animal studies.

This thesis focuses on the development of *in vitro* quality tests for diphtheria toxoid. The general aim is to characterise the antigen as completely as possible by immunochemical and physicochemical means. The obtained data and insights will be utilised to define criteria that diphtheria toxoids have to meet for their use as diphtheria vaccine. Ultimately, the most suitable assays should be validated for the use in routine quality control and replace the *in vivo* tests.

Diphtheria

In 1821, the clinician-pathologist Bretoneau described for the first time the clinical characteristics of diphtheria: a sore throat, membrane production and death by suffocation (1). The bacillus Corynebacterium diphtheriae, the pathogenic organism responsible for diphtheria, is normally found in the upper respiratory tract of the patient. In the serious stage of the disease, a pseudo-membrane is formed by local tissue necrosis, which may cause suffocation and characteristic swelling of the neck, also called a bull-neck (2, 3). The disease can also damage a variety of organs, such as the heart, liver, lungs and kidneys. The diphtheria toxin produced by the bacteria is the predominant cause of these clinical manifestations (2).

Despite immunisation programs in many countries, diphtheria remains a serious health problem in several regions of the world (Eastern Europe, Southeast Asia, and South America) (4). In the last decade, a new epidemic breakout of diphtheria in Russia resulted

in more than 140,000 disease cases and 4,000 deaths, as reported to the World Health Organisation (5). Diphtheria is seldom diagnosed in the Netherlands. In the last fifteen years, the National Institute for Public Health and the Environment (RIVM) has registered only three cases. Probably, the high degree of vaccination contributes to the low incidence in our country.

Diphtheria toxin

One of the first successful developments in pathological investigations of diphtheria is the microscopic study of Klebs who described in 1883 the pathogenic bacterium (2). One year later, Loeffler reported the isolation of the diphtheria bacillus from a pure culture (2). Loeffler could also induce the disease in guinea pigs, by infecting them with the cultured pathogen. In 1888, Roux and Yersin demonstrated that bacterial culture filtrates contain a toxic protein - diphtheria toxin - that is able to kill guinea pigs. In addition, non-pathogenic strains of *C. diphtheriae* were isolated. These strains became pathogenic when they were infected with the specific corynephage-β (6, 7). In 1951, Freeman et al. revealed that the gene of diphtheria toxin is located on this bacterial virus (7). In 1983, the sequence of the structural gene for diphtheria toxin was reported by Greenfield et al. (8).
Diphtheria toxin is one of the most extensively studied and well-understood bacterial toxins (9). It belongs to the group of AB toxins because of its structural organisation (10). Other members of the bacterial AB toxins are, for example, tetanus toxin, pertussis toxin, cholera toxin, anthrax toxin and *E.coli* heat-labile toxin. The B-fragment of all these toxins delivers the A-fragment to the cytosol, causing cell death or affecting the cellular physiology. Diphtheria toxin is secreted as a proenzyme of 58.3 kDa. This proenzyme becomes enzymatically active after cleavage into two fragments (the A-fragment, 21.2 kDa, and the B-fragment, 37.1 kDa) that are still connected to each other by a disulphide bridge (Figure 1) (9). The crystal structure of diphtheria toxin was revealed in 1992 by Choe et al. (11). It

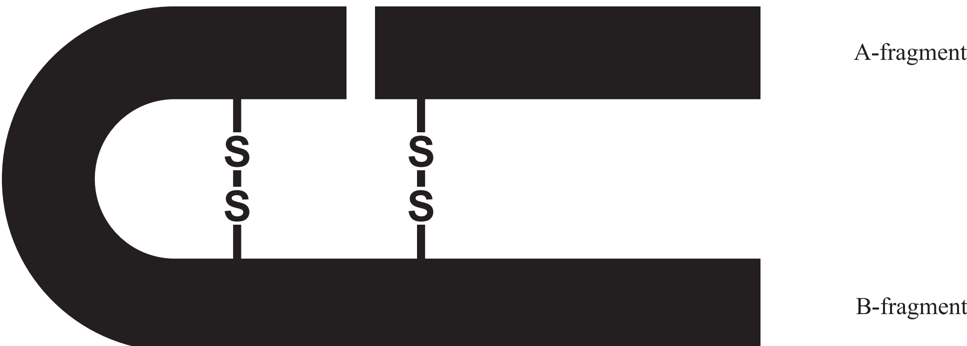

Figure 1. Schematical representation of diphtheria toxin. The enzymatically active A-fragment is linked via a sulphide bridge to the B-fragment, which is involved in the entrance of the host cell.

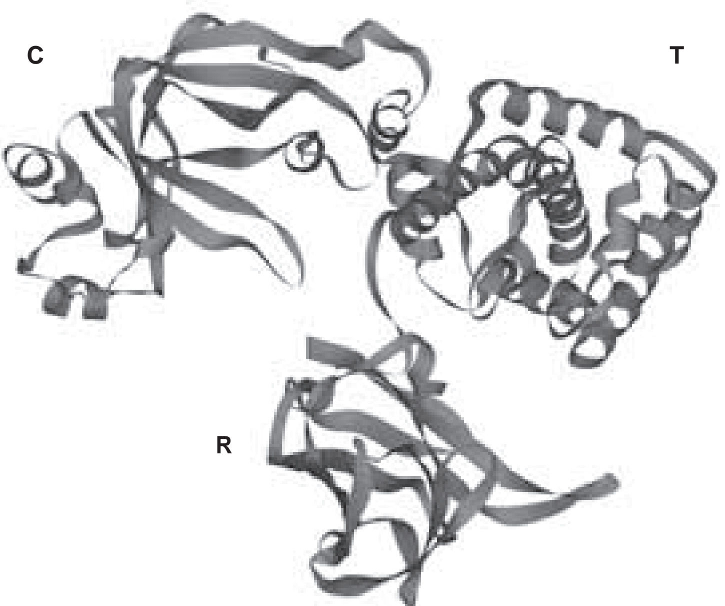

Figure 2. Crystal structure of diphtheria toxin. The three domains of diphtheria toxin are the catalytic domain (C), receptor domain (R) and translocation domain (T). The A-fragment consists of the catalytic domain, whereas the B-fragment contains the receptor domain and the translocation domain (11).

has three discrete folding domains (Figure 2): the catalytic (C-domain), the translocation (T-domain) and the receptor-binding domain (R-domain). The A-fragment corresponds with the catalytic domain, and the B-fragment comprises the translocation and receptor domain. Each domain in the toxin molecule performs a specific role. The biological action of diphtheria toxin starts with the binding of the receptor domain to the diphtheria toxin receptor (Figure 3). This receptor is identical to the heparin-binding epidermal growth factor precursor (HB-EGF) (12). The binding to the receptor triggers endocytosis of the toxin. After endocytosis the diphtheria toxin ends up in lysosomes. The translocation domain penetrates in the membrane of the lysosome due to a conformational change of the diphtheria toxin as a result of the acidic lysosomal environment, and translocates the catalytic domain into the cytosol. The catalytic domain has a binding site for NAD^+ and transfers the ADP-ribose moiety of NAD^+ to elongation factor-2. As a result of the modification of elongation factor-2, the protein synthesis in the host cell is irreversibly inhibited leading to cell death.

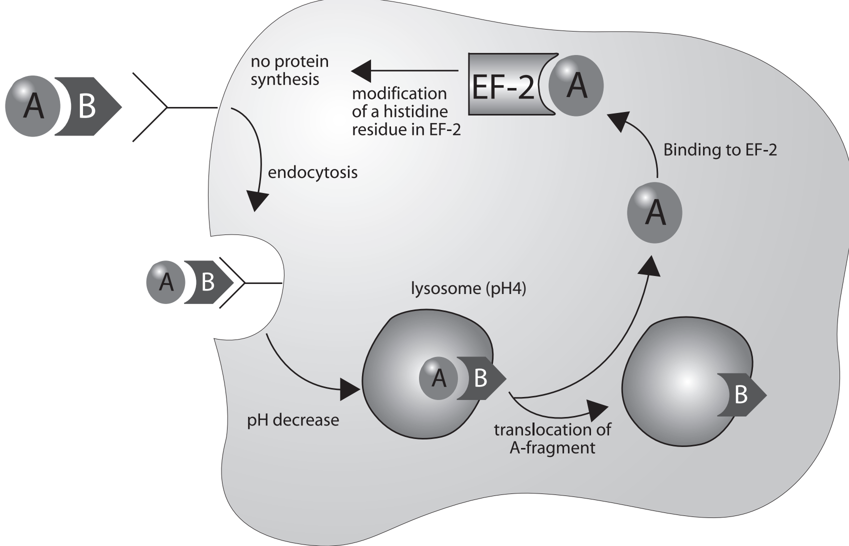

Figure 3. Diphtheria toxin uptake by a host cell (e.g. human or guinea pig) and its biological action. Diphtheria toxin binds to the cell surface receptor HB-EGF through its receptor domain, and is endocytosed and addressed to the lysosome. The translocation domain penetrates in the lysosomal membrane as a result of low pH. The catalytic A-fragment is translocated to the cytosol and inactivates the elongation factor 2 (EF-2)

Diphtheria toxoid vaccine

A cornerstone in the therapeutic treatment of diphtheria is the use of diphtheria antitoxin. This hyperimmune antiserum is produced in horses. In 1891,Von Behring successfully applied antitoxin in a child as a diphtheria remedy. The therapy has shown in a clinical trail to reduce mortality from 7 to 2.5 percent (1).

The approach that has drastically reduced the frequency of diphtheria is vaccination. In the 1920s, Ramon in France, and Glenny and Hopkins in England developed a successful method to prepare a vaccine from a diphtheria toxin. The toxin was incubated with formaldehyde yielding a non-toxic but still immunogenic toxoid (13, 14). Although the production process differs from manufacturer to manufacturer, the current diphtheria vaccine production is essentially still based on their method. Diphtheria vaccine production starts with culturing of a *Corynebacterium diphtheriae* strain until the diphtheria toxin concentration in the culture medium reaches a plateau level. Next, the bacteria are removed and the toxin-containing medium is concentrated. Glycine and formaldehyde is added and the mixture is incubated for several weeks. After the inactivation process, the diphtheria toxoid is concentrated and purified against a saline solution to remove medium components.

The bulk product is diluted until the desired concentration is reached as determined by the flocculation test (15, 16). Finally, diphtheria toxoid is adsorbed onto aluminium phosphate in combination with other antigens, such as tetanus toxoid and inactivated polioviruses (DT-IPV) or with tetanus toxoid, whole cell pertussis bacteria or the acellular equivalent, and inactivated polioviruses (DTP-IPV).
In the Netherlands, children are vaccinated with DTP-IPV at the age of two, three, four and eleven months. At the age of four years, children are boosted with the DT-IPV vaccine.

Outline of this thesis

The inactivation of diphtheria toxin is a critical step in the production of the vaccine. Formaldehyde/glycine treatment affects the structure of the antigen by chemically modifying amino acid residues and changing the protein conformation. This results in the destruction of antigenic sites. The formaldehyde-induced modifications in diphtheria toxin are poorly understood and depend on the reaction conditions, including purity and concentrations of the reactants. In successive productions, variations may occur in a number of production steps, such as the time of culturing, the toxin concentration for the detoxification, the composition of detoxification matrix, and the amount of glycine added as a reactant. This implies that the potency of the resulting toxoid is not necessarily the same between batches. Therefore, the quality of each new-produced toxoid batch is assessed in a potency test with animals.

The primary aim of this project is to characterise the structure of diphtheria toxoid with physicochemical and immunochemical techniques. These techniques will enable us to measure accurately the quality of diphtheria toxoid batches, instead of using animal tests. The modifications that result from the formaldehyde treatment were investigated for a better understanding of the detoxification process. This knowledge may help to develop new *in vitro* tests, as an alternative for the current *in vivo* potency test. Summarising, we focus in this thesis on (i) the identification of critical physicochemical and immunochemical properties of diphtheria toxoid which are predictive for is quality as vaccine; (ii) the elucidation of formaldehyde-induced modifications in diphtheria toxoid preparations; and (iii) the characterisation of the immunodominant epitopes in diphtheria toxin.

In **Chapter 2,** the current status of analytical methods for quality control of human vaccines is reviewed. Today, the quality control of classical vaccines largely relies on animal studies. On the other hand, a number of examples show that analytical tests enter the field of vaccine quality control, especially with regard to the ‘well-defined’ vaccines.

Chapter 3 describes a study of experimental diphtheria toxoids that are characterised with a set of physicochemical and immunochemical techniques. The study yielded six criteria that diphtheria toxoids have to meet: (i) shifted B-fragment on SDS-PAGE gels; (ii) reduction of primary amino groups compared to diphtheria toxin; (iii) increased molar extinction difference at 275 nm and (iv) an unaltered secondary structure as determined by CD analysis; (v) higher resistance to denaturation; (vi) and reduced binding to monoclonals Dim27 and Dim33. The study clearly demonstrates the correlation for these toxoids between the results of *in vitro* analytical methods and those of the potency test performed in mice.

In **Chapter 4**, these techniques are applied to characterise regular diphtheria toxoids from different companies, real-time aged products and material from an adapted inactivation procedure. These products were judged on the basis of the six criteria. The quality predicted by the analytical assays paralleled the *in vivo* potency test.

As a result of the detoxification, diphtheria toxin is converted into a very heterogeneous

toxoid in which the location, the number and the nature of the modifications are unknown. A better understanding of the mechanism and reaction products may help to develop new *in vitro* tests. The formaldehyde-induced modifications are investigated with rationally designed synthetic peptides. Mass spectrometry turned out to be a powerful tool to identify chemical modifications of amino acid residues in peptides and proteins.

Chapter 5 provides an overview of all modifications that result from the formaldehyde treatment, based on a study with model peptides. The study shows a great diversity of modifications, depending on the nature of individual amino acid residues, the peptide sequence, and the composition of the reaction mixture. The information obtained with these model peptides was utilised to study the complex formaldehyde-protein chemistry in the model component bovine insulin and, finally, in diphtheria toxoid itself. This work is described in **Chapter 6 and 7**, respectively.

Diphtheria toxin possesses at least five distinct epitopes as established with monoclonal antibodies. **Chapter 8** describes the selection of mouse monoclonal antibodies, which can be used in panels of immunochemical assays for the quality control of diphtheria vaccines. The selected antibodies have toxin-neutralising capacity and recognise the immunodominant epitopes in diphtheria toxin.

Finally, the thesis is summarised and the perspectives are discussed in **Chapter 9**.

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Chapter 2

Reduction of animal use in human vaccine quality control: opportunities and problems

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Abstract

In vivo assays play a crucial role in the assessment of the potency and safety of human vaccines. Robust vaccine production procedures, improved characterisation methods and development of well-characterised vaccines create possibilities to reduce animal use. In this paper the current status in this field is reviewed. Achievements with regard to *in vivo* and *in vitro* potency and safety testing are discussed as well as new developments and possibilities in the field of *in vitro* characterisation of vaccine components. Finally, validation and implementation issues will be dealt with. Although replacement of *in vivo* tests for batch release of existing vaccines is difficult, emerging technologies allow well-considered reduction of *in vivo* experiments during product and process development and improvement. Inextricably bound up with this approach is Good Manufacturing Practice, resulting in robust, validated production processes.

Introduction

Animal tests play a crucial role in research and development (R&D) and in routine quality control of human vaccines. Quality control of vaccines, *in vivo* as well as *in vitro*, is important in all stages of vaccinology: (i) during R&D, (ii) during routine production (in-process control) and (iii) after production (batch release).

(i) During the R&D phase, *in vivo* experiments cannot be avoided totally. Before phase I studies can commence, the immunogenicity and, if possible, the potency (see Table 1 for definitions) and safety are always determined in animals.

(ii) The quality of vaccines is increasingly guaranteed by the use of robust and reproducible production processes. Regulatory agencies, e.g. the Food and Drug Administration (FDA) and the European Directorate for the Quality of Medicines (EDQM) – European Pharmacopoeia (EP) generate recommendations and guidelines to ensure minimal (but high) quality levels (1). Starting materials, like seed lots and culture media, as well as products are subject to a number of tests to ensure the predetermined quality. Seed lots must not contain micro-organisms other than the seed strain. Culture media are as far as possible free from ingredients known to cause toxic, allergic and other undesirable reactions in man. Critical steps in the production process are monitored with in vitro in-process controls to demonstrate consistency of production.

(iii) For release of the final lot, product quality is investigated. The identity, sterility, stability, safety and potency of the vaccine are determined by validated tests. Batch release is performed both by the manufacturer and the national control laboratory of the country where a particular vaccine batch will be used.

Table 1. Definition of terms related with quantity and quality of vaccine components.

	Definition	Influenced by or dependent on
Quantity	Molarity	Concentration
		Antigen integrity (intact or degraded)
Antigenicity	Ability to bind to specific antibodies	Quantity
		Antigen conformation
		Epitope exposure (epitope density, epitope shielding)
		Matrix (salts, detergents)
Immunogenicity	Ability to induce a specific immune response	Antigenicity
		Species (*in vivo*)
		Status of species (immunogenetic background, age, sex)
		Cell type (*in vitro*)
Potency	Ability to induce a protective immune response	Immunogenicity
		Susceptibility to the pathogen
Efficacy	Ability to prevent disease in the target species	Potency
		Contacts (type, number) within immunised population
		Health status of immunised population
		Infection pressure by target pathogen
		General infection pressure

Adapted from Hendriksen et al. (127).

Table 2. The 3R's as defined by Russell and Burch (2).

Replacement	Substitution of insentient material for conscious living higher animals
Reduction	Reduction in the numbers of animals used to obtain information of given amount and precision
Refinement	Decrease in the incidence of severity of inhumane procedures applied to those animals which still have to be used

In 1959 the book 'The Principles of Humane Experimental Technique' was published by Russell and Burch (2). Although only recognised as such in the late 1970s, this publication was a milestone in animal experimentation resulting in the 3Rs-concept: Replacement, Reduction and Refinement of animal tests (Table 2). This concept is in Europe the framework of existing regulations on animal experimentation (3).
Animal tests are being used in all stages of vaccine manufacturing, from seed lot and cell bank testing through final lot (1). On a yearly basis approximately 1.5 million animals are being used in Europe for routine quality control of vaccines, which is about 10-15% of the total use of animals for biomedical research.
The number of animals used for quality control of vaccines can be reduced drastically if vaccines become better characterised. The FDA uses the definition of a well-characterised biological to determine which product may be exempted from lot-by-lot release (4). Currently, the definition of a well-characterised biological is: a chemical entity whose identity, purity, impurity, safety, potency, and quality can be determined and controlled (5). Each parameter must be identifiable and quantifiable. The virtue of a well-characterised product is that measurements relate structure to function. If correlation between a range of chemical and biological approaches to the biological is demonstrated, potency testing in animals can be replaced by a corresponding assay. Besides a well-characterised product, a validated and reproducible production process is essential for cancelling animal tests for batch release. A consistent process ensures that batches are comparable and can be substituted. The trend has been to develop consistency in manufacturing process by following rules given by Good Manufacturing Practices (GMP) (6). This, ultimately, will allow characterisation of these products by a set of *in vitro* methods rather than by *in vivo* methods.
Many vaccines used today are not well-characterised biologicals. Therefore, batch release will remain mandatory. Nevertheless, it should be possible to reduce *in vivo* testing during process development and even during batch release procedures. In this review, possibilities and progress with regard to alternatives for *in vivo* testing and the problems associated with the introduction of alternatives will be discussed.

Potency testing

Legal requirements mandate that each batch of vaccine produced should be tested for its potential to induce protective immunity after administration. There is a fundamental

difference in the design of a test between live and inactivated vaccines. In the case of live, attenuated vaccine material (for example Bacillus Calmette-Guerin (BCG) vaccine and oral polio vaccine (OPV)), the efficacy of each vaccine batch is related to the number of live particles, determined either by counting or by titration, that is, entirely *in vitro*. *In vivo* testing is only carried out in case of a new seed strain.
Unlike live vaccines that are quantified by *in vitro* titration, an *in vivo* potency test is required for each batch of inactivated vaccines. A number of exceptions exist, such as the SRD (single radial immunodiffusion) potency test for the influenza vaccine and the ELISA (enzyme-linked immunosorbent assay) for genetically engineered hepatitis B vaccine. Generally, potency of a human vaccine is assessed in an immunisation-challenge test using small rodents (mice or rats) as the experimental model. Depending on the type of vaccine, different endpoints are used, such as death/survival ratios (whole cell pertussis, diphtheria toxoid and tetanus toxoid, rabies vaccine), clinical signs (diphtheria, tetanus) or colonisation (whole cell and acellular pertussis). By establishing a dose-response curve in parallel to a standard preparation with known potency in international units (IU/ml), the potency of the vaccine is expressed in units of the standard preparation, including 95% confidence interval. A challenge model is not always available. In those cases potency testing is limited to serology: after immunisation the antibody response is measured. At least part of the functionality of the antibodies can be determined by their ability to neutralise the pathogen in vitro or to their ability to kill bacteria in the presence of complement.
Several developments have occurred recently, which will lead to a reduction and/or refinement in the use of animals. To mention a few examples:

(i) There is now consensus that the quantitative information obtained by parallel-line assays is overdone for vaccines such as tetanus toxoid. Pharmacopoeias only state that estimates of potency should exceed a minimal level of international units. Furthermore, these vaccines are consistently produced and potencies of these products generally exceed the minimal level of potency substantially. Therefore, steps are being taken to replace the multi-dilution test by a simplified test, in which only one dilution of the vaccine under study is compared with one dilution of the reference vaccine and that only allows us to demonstrate that the vaccine potency is above a certain level (Table 3).
(ii) For some vaccines, including the toxoids, serological alternatives have been developed for the challenge procedure. Thus, for diphtheria and tetanus toxoid vaccines potency might no longer be determined by means of a challenge, but by bleeding of the animals under anesthesia followed by serum titration in tissue culture (e.g. diphtheria vaccine) or by another antibody assay system such as ELISA (e.g. tetanus vaccine) (Table 3). International validation studies have shown the validity of this approach for tetanus toxoid vaccines (7-9).
(iii) The European Pharmacopoeia and Code of Federal Regulation now allow for the use of 'humane end-points' as an alternative to the lethal parameter. Several guidelines exist

Table 3. Reduction and refinement of animal use in diphtheria and tetanus toxoid potency testing.

	Test method (read out)			
	Challenge (protection)		Vero (diphtheria) or ToBI[b] (tetanus) (antibodies)	
	Multi-dilution	Single-dilution	Multi-dilution	Single-dilution
Diphtheria toxoid	101 guinea pigs	32 guinea pigs	64 mice	20 mice
Tetanus toxoid	108 mice or 102 guinea pigs	32 mice or guinea pigs	64 mice[b]	20 mice[2]

[a] ToBI: toxin binding inhibition assay.

[b] Same mice can be used for both diphtheria and tetanus toxoid testing.

Adapted from Milstien et al. (10).

concerning humane end-points (10). The E.P. states that: "…if it is indicated that an animal is considered to show positive, infected etc. when typical clinical signs or death occur then as soon as sufficient indication of a positive result is obtained the animal in question shall be humanely destroyed or given suitable treatment to prevent unnecessary suffering." Thus, for potency testing of whole cell pertussis vaccines certain clinical signs and the fall in body temperature can now be used as an end-point rather than death (11) and in the case of rabies vaccine clinical signs and a loss of body weight (12) can be used as an end-point.

Of a more fundamental nature is the question of whether the current potency tests do justify the extensive use of laboratory animals. It is not uncommon to find large variations in results between laboratories, most probably due to the genetic differences of the animals used (13). So, most potency tests suffer from a poor reproducibility. In addition, the relevance of the animal models for the human situation has been challenged. Finally, animal testing is becoming very expensive and will become even more expensive in the near future. This, and the consideration that also conventional vaccines are now better-defined products (e.g. thanks to GMP and advances in analytical methodology), have prompted the interest in the application of *in vitro* techniques for product characterisation (see later section: The fingerprint approach).

Safety testing

Safety, not potency, is perhaps the most important requirement for a vaccine. Adverse effects can have a strong negative effect on vaccine coverage, because the benefits of vaccination are not directly clear for individual vaccinees, whereas adverse effects are immediately apparent. Vaccine safety is maintained by developing consistent production processes, extensive safety testing and post-marketing surveillance. In general, parenteral administration of a vaccine causes local pain and swelling at the injection site. Often a mild fever is observed. To keep these effects within acceptable limits, pyrogenicity tests are performed (see later paragraphs).
However, safety tests are also done to prevent more serious adverse events. Potential safety

problems of attenuated vaccines are reversion to wild type. The potential risk of inactivated vaccines is that they are not killed or detoxified completely. This has caused several accidents, like the Cutter incident in 1955 in the U.S. Five people died as a result of incompletely inactivated polio vaccine. After this accident, rules for safety became more stringent (14). Many safety tests are performed *in vivo*. Later, the developments and possibilities of alternative tests are discussed.

The abnormal toxicity test

Abnormal toxicity is the EP nomenclature (1). The test is also called general safety by the United States Code of Federal Regulations (15) and innocuity by the World Health Organization (WHO) (16). The purpose of the test is to detect any toxicity of abnormal contaminations. Especially in the last decade, the status of the test has changed (17, 18). The abnormal toxicity test for human vaccines, according to the EP, is only required if a specific toxicity test is not available, e.g. for typhoid vaccine, cholera vaccine, Haemophilus influenza type B-conjugate (Hib), pneumococcal and meningococcal polysaccharides, and influenza vaccine. For one test, the EP requires five mice and two guinea pigs, injected with one human dose. The preparation passes the test if none of the animals shows signs of illness. The distress for the animals is generally slight (19). However, the value of the test is questionable (19, 20), because the small number of animals makes detection of contamination unlikely (20-22).

No *in vitro* model for the abnormal toxicity test is available. The European Centre for the Validation of Alternative Methods (ECVAM) Workshop (23) recommended to WHO to omit the present animal test for abnormal toxicity or to perform the test on the final bulk instead of final lot (10). In some cases, several lots are produced with one bulk. As an obvious result, the number of tests is thus reduced. Risk of toxic contamination is extremely low if manufacturers comply with GMP rules and if consistency in production is guaranteed (10, 19).

Specific toxicity tests, absence of toxicity, irreversibility of toxicity

A number of vaccines contain antigens that are inactivated bacterial exotoxins. The specific toxicity test is used as a final check for the inactivation. Depending on the vaccine type, the test is performed in guinea pigs or mice. EP monographs are available for toxoids and pertussis vaccines.

Alternative tests to measure residual toxicity in toxoid vaccines are available. The Vero cell is very sensitive to diphtheria toxin and therefore suitable to detect minute amounts of residual toxin in purified toxoid (24-26). A validated Vero cell test can replace the *in vivo* test for diphtheria vaccine according to the WHO requirements (16).

Another realistic alternative for the specific toxicity test is measurement of the enzymatic activity of toxin. Biochemical assays have been described for tetanus and pertussis toxin (27, 28). Synthetic, fluorescent peptides mimicking the natural substrate are used.

Degradation products are quantified by HPLC with fluorescence detection. The tests are very specific and sensitive. Validation studies should demonstrate whether these assays have potential to replace *in vivo* testing (29).
For whole cell pertussis vaccines, the mouse weight gain test is difficult to replace because B. pertussis produces several toxins (30). A modified mouse weight gain test was developed, making it possible to discriminate between effects of endotoxin (weight loss after 16 h after immunisation) and effects of pertussis toxin (leukocyte counts after 7 days) (31). In addition, toxicity testing and immunogenicity testing could be combined.

(Neuro)virulence tests
The potential danger of live attenuated vaccines is that they might revert to virulence. Seedlots of live viral vaccines and BCG are verified for reversal. Final lots of the oral polio vaccine are also checked for neurovirulence. Virulence tests are currently performed in monkeys. The use of primates urges to look for alternatives. Besides, these tests sometimes have a limited discriminative power, as was demonstrated for mumps vaccine (32). For oral poliovirus, two alternative assays are available or developed (33): a transgenic mouse neurovirulence model and a genetic *in vitro* test.
In the late 1980s transgenic mouse strains were developed, expressing the human poliovirus receptor (34, 35). Like primates, these mice can be infected with all three polio serotypes and they develop clinical signs and lesions in the CNS (central nervous system). For type 3, the serotype that is most difficult to control, a neurovirlence test was developed and, after an international collaborative study, adopted by WHO as an alternative for the monkey model (36).
A second alternative is an *in vitro* test based on detection and quantification of mutations in the genome. This approach is feasible, because reversion to virulence is a result of minor mutations (33). Mutant analysis by PCR and restriction enzyme cleavage (MAPREC) (37) involves: (i) RNA extraction of the vaccine; (ii) reverse transcription and cDNA amplification by PCR, (iii) restriction enzyme cleavage; (iv) separation of fragments and quantification of bands on gel. For all three serotypes the MAPREC test is available. For type 3, MAPREC results correlate well with neurovirulence. For type 1 and 2 the correlation is less evident. Although MAPREC is not yet a replacement test for neurovirulence, the method is used as a preliminary test prior to *in vivo* testing (type 3) or to demonstrate consistency of production (type 1 and 2). Similar molecular tests are now under investigation for mumps and yellow fever vaccines (38).

Test for pyrogens
Pyrogens form a heterogeneous group of substances that induce fever. Cell wall components of Gram-negative bacteria, consisting of endotoxins (lipopolysaccharides) form an important class of pyrogens. However, many pyrogenic agents have not been identified. Therefore, the test for pyrogens is still performed *in vivo* for some vaccines. The test

described by the EP requires three rabbits in which body temperature is monitored for three hours after injection of saline and then for at least three hours after injection of the product. The test is not a general requirement for all vaccines. It is mandatory especially for polysaccharide vaccines, but also for rabies and tick borne encephalitis vaccines. *In vitro* alternatives are (becoming) available. The *Limulus amoebocyte* lysate test (LAL test) (39) is based on endotoxin initiated coagulation in a lysate prepared from the blood of the horseshoe crab (*Limulus polyphenus*). The lysate reacts specifically with bacterial endotoxins. The sensitivity of the LAL test is similar to that of the rabbit test (40). This test is accepted by the EP as test for bacterial endotoxins and is applied for hepatitis A, Hib, influenza, rabies, typhoid and yellow fever vaccine. Although invertebrate animals are required to obtain blood, the procedure is generally not lethal.

A (new) pyrogen assay using human blood is under development (41, 42). Blood contains monocytes and macrophages that release mediators if they have been into contact with pyrogens. An ELISA is used to determine formation of interleukin-1β. The assay seems to be less sensitive than the LAL test. However, the assay is not restricted to endotoxins: it can detect a broader spectrum of pyrogens. A disadvantage is that fresh blood is necessary, because after 8 hours storage at room temperature there is spontaneous release of interleukin-1β.

For the detection of lipopolysaccharides, SDS-PAGE with silver staining and gas chromatographic quantification of 2-keto-3-deoxyoctonate or fatty acids after hydrolysis of the lipopolysaccharides can be used (43, 44).

Extraneous agents

The test for extraneous agents is required to detect viral contaminations in virus seed lots, virus harvest, cell culture and control eggs. Only virus seed lots are tested in adult and suckling mice and guinea pigs, other materials are tested in cell cultures. An amount of the seed lot is neutralised by virus specific antibodies and then injected. The virus seed lot passes the test if no animal shows evidence of infection. The nature of the test, detection of unknown contaminations, makes it difficult to develop *in vitro* alternatives. When the nature of possible viral contaminations in animal cell cultures are known, assays based on PCR are alternatives (45, 46).

The fingerprint approach

The development and validation of *in vitro* tests that correlate with potency is a tedious and complex process and is rarely successful. A thorough knowledge of the immune response after infection or vaccination is essential. This knowledge is often not or only partially available. An approach that less heavily relies on understanding of mechanistic effects is the demonstration of batch comparability. This method assumes that if vaccine batches are

produced consistently, these batches must have identical properties *in vivo*. The question here is of course: when are two vaccine batches equivalent and which vaccine characteristics are relevant to demonstrate comparability? In general the potency and safety of vaccine batches are determined by antigen structure and vaccine composition. The possibilities to obtain information about structure and composition have expanded enormously in the past decades (Tables 4-6). Some analyses may provide information about the functionality of the vaccine, i.e. quantitation of the binding of a neutralising or bactericidal monoclonal antibody, but in many cases correlation with potency is limited, absent or unknown. Nevertheless, structural information as such may prove very useful. To draw sensible conclusions from 'non-functional' assays, it is a prerequisite that a battery of tests is applied. In general, no single analytical technique can completely characterise an antigen. The use of several non-related techniques results in a fingerprint of the antigen. This fingerprint is then compared with a reference preparation with proven potency or with criteria that are determined during the development phase of a (new) vaccine.
This approach is not (yet) suitable for routine batch release of classical vaccines, because they have a complex, partially unknown composition and/or structure, i.e. they are not well-characterised biologicals (see Introduction for definition). In the process development phase 'finger printing' can be very valuable, especially when an existing production process is changed and comparability has to be demonstrated. Changes in production procedures (new culture media, adaptation of purification methods, scaling up, etc.) require from vaccine manufacturers that they demonstrate that the vaccine obtained with the adapted production process has the same quality as compared to the 'old' process. Fingerprinting the vaccine is a way to measure the degree of comparability. This information can be used to rationalise decisions about the necessity of clinical tests.
The presence of the extrinsic factors (e.g. other antigens or adjuvants) is a major hurdle in the replacement of *in vivo* tests. Ideally, this should be achieved by the development of functional *in vitro* tests that mimic *in vivo* immune responses (see later paragraphs). Up to now the development of such tests remains very difficult. However, it should be possible to predict and guarantee the potency of final products by a combination of accurate antigen quantification in the bulk material, use of consistent production methods (monitored by adequate in-process controls) and a number of *in vitro* quality control tests without performing an *in vivo* potency test on the final product. The use of better-defined antigens increases success chances of this approach. For instance, if tetanus toxoid batches can be produced consistently and if these batches are indistinguishable from each other (with respect to purity, toxoid structure and, as a result, antigenicity), the potency of alum-adsorbed final lot is reflected by the concentration (expressed as Lf-units) of the non-adsorbed toxoid bulk, provided that the aluminium adjuvant is produced consistently and that the adsorption procedure is performed consistently. This implies of course that thorough characterisation of the aluminium adjuvant is as important as that of the antigen.
A brief overview of available techniques and their applications in vaccinology is given later

Table 4. Immunochemical methods for *in vitro* characterisation of antigens and vaccines.

Technique	Principle	Suitable for / information about	Pros	Cons	Selected examples
Nonlabelled immunoassays (immunoprecipitation, flocculation)	Formation of visual immune complexes at equivalent concentration of antigen and antibody	Quantification; flocculation time provides additional information on toxoid quality	No expensive equipment needed; easy to perform; cheap	Low precision; subjective read out (technician's eye)	Diphtheria and tetanus toxoid (128, 129)
ELISA	Solid state binding in 96-well plates	Quantification; epitope quality	Easy to perform; cheap; sensitive	Sometimes low precision	HBsAg (115), IPV (130), Tetanus toxoid (131)
Biosensor	Antibody-antigen interaction is measured on a custom sensor surface by SPR or resonant mirror	Quantification; epitope quality; kinetic analysis	High precision; binding is observed momentarily; affinity measurements relatively easy	Expensive; special expertise required; less suitable for very small and very large antigens	HBsAg (132), IPV (133), HAV (134), HIV (135), Diphtheria toxoid (88, 136)
Immunoblotting	Size or charge based electrophoretic separation, followed by immunodetection	Antigen identification and antigen integrity (fragmentation); antigen specificity of antisera	Easy to perform; cheap; sensitive	Antigen is detected in denatured form; qualitative test	HIV (75), PorA (96), PorA, antisera (95)
Immunogold electron microscopy	Epitope visualisation by 'staining' with antibody coated gold particles (ca.10 nm)	Epitope localisation and exposure	Association of epitope with larger structure (virus, vesicle) can be demonstrated	Expensive equipment required; prone to artefacts	PorA (96)

Abbreviations: HBsAg: hepatitis B surface antigen; IPV: inactivated polio vaccine; HAV: hepatitis A virus; HIV: human immunodeficiency virus; PorA: pore protein A from N. *meningitidis*.

Table 5. Analytical chemical methods for *in vitro* characterisation of antigens and vaccines.

Technique	Principle	Suitable for / information about	Pros	Cons	Selected examples
Peptide mapping	Enzymatic or chemical degradation followed by separation of the resulting peptides	Protein modifications; stability (proteolytic degradation)	Sensitive; detection with HPLC and MS	Only usable for proteins; antigen mixtures cannot be analysed; Standardisation difficult (different per protein)	CRM_{197} (66), OspA (67), HepE protein (67). *H. pylori* antigens (65)
Mass spectrometry	Mass/charge detection of ionised molecules in magnetic or electric field or by flight time	Mass, identity; integrity, stability; protein modifications	Very sensitive; detailed structural information obtained	Expensive equipment; special expertise required; gas phase analysis only; ionisation required; salts interfere	Anthrax PA (137), pertussis toxin (138), CRM_{197} (66), HepE protein (64), OspA (67)
Colourimetric assays	Analyte is chemically modified resulting in colour change	Quantification of carbohydrates, phospholipids, free amino groups in proteins	Easy; cheap	Low specifity	Free amino grops (76, 77) polyaccharides: ribose, inorganic phosphate, sialic acid (73, 74) neutral sugars (75)
Chromatography					
Size exclusion	Size based separation; large molecules do not penetrate pores in the stationary phase, resulting in fast elution. Smaller molecules are retained	Hydrodynamic size, purity, stability (aggregation)	Easy, non-denaturing	Low resolution, sample volume max. 10% of column volume (high concentration), relatively long run time, column quality critical (no voids)	Polysaccharide-conjugates (78-82, 90), HAV (84), toxoids (83, 85-89)
Ion exchange	Charge based separation; binding at low ionic strength, elution with salt or pH	Purity; protein modifications; polysaccharide quantification; amino acid composition	Relatively easy; generally non-denaturing	Standardisation difficult (conditions different per antigen)	Polysaccharides (73, 80, 82, 90-94), amino acids (51, 67, 87, 89)
Reversed phase	Hydrophobicity based separation; binding at low organic solvent %, elution with organic solvent	Purity; protein modifications; stability; digest analysis	Relatively easy; can be combined with MS detection	Denaturing; standardisation difficult (conditions different per antigen)	See digest analysis, Anthrax PA (139)
Hydrophobic interaction	Hydrophobicity based separation; binding at high ionic strength, elution with low ionic strength	Purity; protein modifications; stability	Relatively easy; non-denaturing	Standardisation difficult (Conditions different per antigen)	
Electrophoresis					
PAGE (SDS or native)	Size or charge (in native mode) based separation; pretreatment with SDS results in negatively charged proteins	Purity; size (idenity); protein modifications; stability (degradation; aggregation)	Easy	Qualitative assay, denaturing (in SDS mode)	*N. meningitidis* OMPs (68, 69), Tetanus toxoid (104), Anthrax PA (139), HBsAg (108)
IEF	Charge based separation; proteins move in a pH gradient to pH~IEP	Purity; IEP (identity); protein modifications; stability	High resolution	Qualitative assay; risk of isoelectric precipitation	Toxoids (87, 89)
Two-dimensional	Combination of IEF (1st dim.) and SDS-PAGE (2nd dim.)	Analysis of complex mixtures	High resolution	Relatively difficult; qualitative assay	OspA (67), BCG (140) , *H. pylori* antigens (65)
CE	Electrophoretic separation in a capillary	Peptide analysis; purity; stability	High resolution; fast separation; can be combined with MS detection	Analysis of proteins is difficult due to interactions with capillary	Diphtheria toxoid (136), Anthrax PA (139)

Abbreviations: CRM_{197} OspA: outer surface protein A (from B. burgdorferi); HepE: hepatitis E; HAV: hepatitis A virus; PA: protective antigen; OMP: outer membrane protein; BCG: *Bacillus Calmette-Guerin*.

Table 6. Spectroscopic methods for *in vitro* characterisation of antigens and vaccines.

Technique	Principle	Suitable for / information about	Pros	Cons	Selected example
Circular dichroism	Differential absorption of left- and right-handed circularly polarised light	Secondary (far UV) and tertiary structure (near UV) of proteins	Relatively easy to perform (measurements); no sample consumption	Not possible to pinpoint conformation changes in the molecule; special expertise required (interpretation)	Toxoids (86, 89, 104, 105), Polysaccharide conjugates (66, 80, 102, 141, HAV (84), *B. pertussis* antigens (85, 142)
Fluorescence	Intrinsic fluorescence (intensity and emission maximum) of proteins after excitation	Protein conformation; protein modification (formaldehyde)	Relatively easy to perform (measurements)	Not possible to pinpoint conformation changes in the molecule; special expertise required (interpretation)	Toxoids (83, 102, 104, 136, 143), Polysaccharide conjugates (82, 102, 141), OspA (99), HAV (103), *B. pertussis* antigens (86)
Infrared spectroscopy	Infrared absorption	Secondary structure of proteins	Solid state analysis possible (freeze-dried materials)	Fluid state analysis difficult; high concentration needed	Tetanus toxoid (107), HBsAg (108)
NMR	'Flip' of magnetically aligned nuclei in a scanning electromagnetic field	Molecular structure of polysaccharides	Detailed structural information obtained	Expensive equipment; special expertise required	Polysaccharide (66, 73, 81, 107, 109-113, 141)
Electron microscopy (see also immunogold EM)	Imaging by ultrashort wavelength radiation (electrons)	Imaging of supramolecular structures; integrity	Shape and structure information obtained	Expensive equipment; special expertise required; qualitative analysis; sample preparation may induce artefacts	meningococcal vesicles (96), HAV (84, 103), HBsAg (115), acellular pertussis vaccine (144)
Dynamic light scattering	Brownian motion is measured by light scattering fluctuations	Particle size (<1μm)	Easy to perform low sample consumption fast	No information about shape and structure	HAV (84, 103), HBsAg (115)

Abbreviations: HAV: hepatitis A virus; OspA: outer surface protein A (from B. burgdorferi); HBsAg: hepatitis B surface antigen.

(see also Tables 4-6). In this review we focus on methods that give information about the structure of antigens. Specific analytical techniques used to determine the concentration of vaccine components other than the antigen(s), like the quantification of aluminium, formaldehyde, nucleic acids, etc., are not discussed.
One can discern three categories of techniques from which structural information can be obtained: immunochemical, analytical chemical and spectroscopic. In addition, *in vitro* immunological methods are being developed.

Immunochemical analyses (immunoassays)
Antigenicity measurements are generally applied to characterise and quantify antigens. By definition, measurement of antigenicity requires the use of antibodies (see Table 1). Their specificity makes them excellent reagents, even in very complex matrices. Today, mainly monoclonal antibodies are used, although not exclusively. Antigenicity in a quantitative sense is often expressed as a measure of concentration. One has to realise, however, that antigenicity and antigen concentration are not necessarily the same.
Many types of immunoassays exist (47-49). Basically, one can discern four groups: radioimmunoassays, enzyme immunoassays (ELISAs), non-labelled immunoassays (flocculation, immunodiffusion, immunoelectrophoresis, etc.) and biosensor analysis. Two of these will be briefly discussed: ELISA because the majority of antigenicity measurements are performed in that format, and biosensor analysis because of its increasing importance in antigen characterisation.

Enzyme-linked immunosorbent assay (ELISA) - ELISAs were developed in the early 1970s as an alternative for quantitative radioimmunoassays (50). They are heterogeneous, i.e. solid-phase enzyme immunoassays. The antigen concentration is calculated relative to a reference with known antigen concentration. Producing a suitable reference preparation, establishing its concentration and assuring its constant quality is difficult and time consuming. Preferably, an international standard is available. Organisations like WHO, EDQM and NIBSC manage these standards. Collaborative studies are organised to assign the concentration of a new batch of an international standard. This standard is used to calibrate the ‘in house’ reference. The difficulties associated with the assignment of the antigenicity of a reference are illustrated with the reference for inactivated polio vaccine (51). Two collaborative studies, organised independently by the EP (52) and WHO (51), did not lead to conclusive results with respect to one of three serotypes. A small reconciliation study was also inconclusive. A second reconciliation study was necessary to establish a definitive antigen content (52). This demonstrates that inter-laboratory results of a test may vary enormously, regardless whether the same protocol and monoclonal antibodies are used or labs are allowed to use their own protocol.

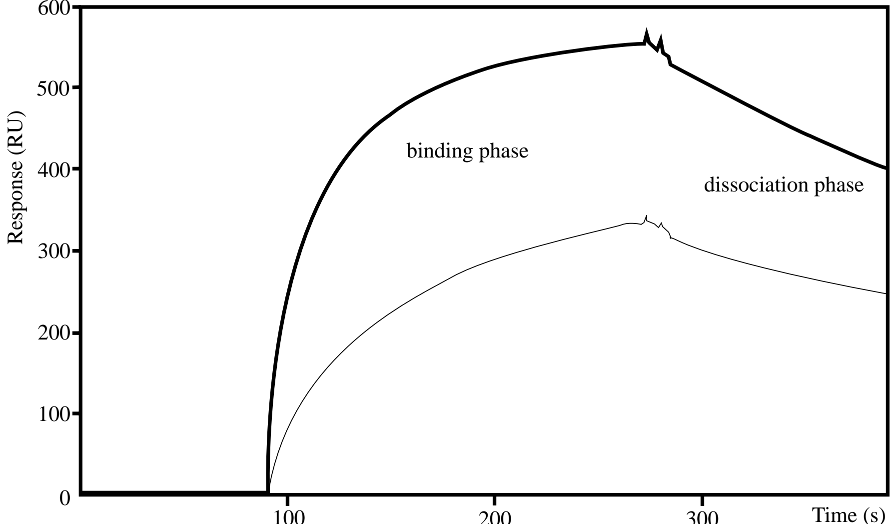

Figure 1. BIAcore sensorgrams. Binding of diphtheria toxin (bold line) and toxoid (thin line) to an anti-diphtheria sensor prepared by covalent coupling of anti-mouse IgG antibodies to the sensor, followed by Fc-binding of an anti-diphtheria toxin MAb (not shown in the sensorgram). On and off rates provide information about the toxoidation process. From Kersten et al. (126).

Biosensor analysis - ELISA is the method of choice to determine antigenicity (Table 1). The result is an antigen concentration that is more or less an average of epitope concentration and epitope quality. With biosensor analysis (53) one can separate epitope concentration from epitope quality (54, 55). As a result, it is possible to develop rapid concentration measurements of antigen and to study epitope quality in a detailed manner. The general principle of biosensor analysis is the binding of macromolecular analytes (protein antigens) to ligands (monoclonal antibodies). The ligand is immobilised on a sensor surface. Subsequently, binding of the analyte is measured. Detection of binding is possible because of changes in the refractive index near the sensing surface and is based on evanescent wave changes. Four types of evanescent field biosensors are commercial available: Biacore (surface plasmon resonance), Texas Instruments Spreeta (surface plasmon resonance), Affinity Systems IAsys (resonant mirror), BioElectroSpec (total internal reflection fluorescence) (56). Binding and dissociation are measured momentarily and continuously, resulting in binding and dissociation curves (Figure 1). Depending on the conditions, the analyte concentration or the affinity constant of the antibody-antigen binding can be determined (57, 58). Very small (peptides) and very large analytes (virus particles, bacteria) are difficult to detect because of low mass and large distance to sensor surface, respectively. Nevertheless, successful applications have been described for large analytes (Table 4). Biosensor analysis has been used to demonstrate batch comparability and to monitor the influence of production changes and storage on product characteristics (Table 4).

Although kinetic data are easily generated, proper interpretation is cumbersome. Since biosensor analysis is commercially available, a lot of progression was made with respect to interpretation and modelling of kinetic data, but pitfalls were clearly demonstrated (53, 59, 60). When the objective is to demonstrate batch comparability this may not be a problem as long as the results are expressed in a relative manner.
Finally, biosensor analysis can be used to get more data from antiserum analysis by measuring the avidity of the antibodies (61). This may reduce the number of *in vivo* experiments in the R&D phase.

Analytical chemical assays
Peptide mapping - A common technique for the quality control of well-characterised biologicals is peptide mapping. The protein is digested with a proteolytic enzyme or by a chemical like cyanogen bromide, and the resulting peptides are separated by HPLC or capillary electrophoresis. The digestion conditions as well as the separation must be optimised for each protein (62). Using a mass spectrometer (see later paragraphs) as detector, the primary structure of the peptides can be deduced and modified amino acids can be identified (63-66). Most vaccine related applications are still restricted to characterise potential vaccine candidates in the research stage (63-65), but the technique has also been used to demonstrate batch comparability of a recombinant Lyme disease vaccine (67) and to characterise CRM197, a non-toxic variant of diphtheria toxin, used as carrier for polysaccharides (66).
A variation on this technique is measurement of trypsin sensitivity of protein antigens, sometimes in combination with denaturing conditions like heat or SDS treatment. An advantage if this type of stability measurements is that degradation can be followed with electrophoresis instead of technically more complicated HPLC or capillary electrophoresis (68, 69).

Mass spectrometry - Mass spectrometry (MS) is increasingly used in the broad field of immunology and vaccinology research (70, 71). It is used for determination of antigen structure, epitope identification, antigen processing (identification of HLA-presented peptides), and quality control of peptide vaccines. The principle of MS includes three steps: ionisation, separation of the formed ions according to differences in mass/charge ratio and detection of the separated ions. Depending on the ionisation technique, the exact mass of proteins of more than 200 kDa can be determined, making MS suitable for identification and determination of integrity (Table 5).
Currently, MS equipment to measure intact proteins is expensive (see Poland et al. (70) for an explanation of the different mass spectrometric techniques). An attractive alternative for intact proteins is the analysis of proteins after enzymatic or chemical digestion. The fragments of the digested protein can be analysed with or without a preceding chromatographic separation. This results in sequence information (64, 65, 67). In this way

MS can be applied for the elucidation of mistranslation sites, point mutations, post-translational and chemical modifications.
Also information on epitope integrity can be obtained, when monoclonal antibodies form immune complexes with the antigen (72). The difference in mass spectra of digested immune complex and the digested antigen reveals information about the epitope. Even discontinuous epitopes can be identified by this technique. Comparative analysis of tryptic digests of polysaccharide carrier protein and conjugate may reveal the location of carbohydrate modifications (66). As the equipment becomes cheaper, smaller and easier to handle, it is expected that MS will start to enter the field of quality control during process development and routine testing. On the other hand, proper experimental techniques and interpretation of mass spectrometric data requires input from specialists.

Colourimetric assays - Colourimetric assays are easy to perform and provide information about the concentration of a number of important vaccine components. The polysaccharide content of H. *influenzae* and N. *meningitidis* type A and C can be determined by colourimetric measurement of the ribose (Bial assay), phosphate and sialic acid content, respectively (73, 74). Neutral sugars, present in glycosylated antigens, are quantified by a phenol-sulphuric acid assay (75).
Primary amino groups present in protein antigens are detectable after modification with trinitrobenzenesulphonic acid (76) or with fluorescamine (77). Amino group quantification is especially useful to monitor the formation of formaldehyde adducts during inactivation procedures. A disadvantage of colourimetric assays is possible interference from other compounds. Therefore, they are less suitable for in-process controls during down stream processing. Alternatives like chromatography have been developed for carbohydrate quantification (see later paragraphs).

Liquid chromatography - HPLC is an established analytical technique for decades and available in almost any laboratory dealing with process development and quality control. Analytes are separated based on their size, charge, hydrophobicity or affinity. Size exclusion chromatography (SEC) is often used to determine the quality and stability of protein antigens, because the technique is straightforward and 'non-interacting' (i.e. the analyte does theoretically not interact with the stationary phase). It is also used for polysaccharide-conjugate vaccines (78-82), but not exclusively. Aggregation behaviour of antigens (83), size changes of viral vaccines upon storage (84) and the effect of formaldehyde treatment of toxins can be monitored by SEC (85-89). The preference for SEC in vaccine quality control does not mean that this technique is superior to other types of chromatography. Ion exchange, reversed phase and hydrophobic interaction chromatography should be considered as alternatives. Oligosaccharide profiling after hydrolysis of sialic acid-containing polysaccharide can be done by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (73, 80, 82, 90-92,

93). For Hib polysaccharide quantification (94) both EU and WHO recommend HPAEC-PAD instead of dry weight determination.

Electrophoretic techniques - SDS-PAGE has been routinely used for several decades for the analysis of protein based vaccine components. Iso-electric focussing (IEF), two-dimensional electrophoresis (a combination of IEF and SDS-PAGE) and capillary electrophoresis are less frequently used for vaccine quality control. Electrophoretic techniques can reveal the same physical properties of protein antigens as liquid chromatography. Protein-protein interactions are detectable by SDS-PAGE if samples are not heated or by titration with increasing amounts of SDS, sometimes combined with limited proteolysis as was demonstrated for meningococcal pore proteins (68, 69). Isoelectric focussing will detect changes in charged groups due to for instance formaldehyde treatment (87, 89). The high resolution of two-dimensional gel electrophoresis is rarely used for quality control purposes (67), but the technique may be useful as in-process control during cultivation of bacteria, which may adapt protein expression as a function of metabolic conditions.
The detection of relevant antigens in gels is facilitated by blotting and subsequent detection with antibodies (Western blotting). It is a qualitative assay, providing information about the presence and molecular weight of antigens. The technique is especially useful for the analysis of samples containing many antigens (75). Immunoblotting is also valuable to obtain more detailed information from *in vivo* experiments (Refinement). In a qualitative manner it is possible to determine the antibody response against particular antigens in a complex mixture (95, 96).

Spectroscopic methods
The intrinsic (i.e. without the contribution of adjuvants) immunogenicity of protein antigens partly depends on their conformation. Recognition of epitopes is for many B-cells conformation dependent. Changes in the conformation as a result of, for instance, variations in process parameters or storage conditions may affect the immunogenicity of a particular protein vaccine. Conformational changes in proteins can be detected by a variety of spectroscopic techniques, such as circular dichroism, fluorescence spectroscopy and infrared spectroscopy. NMR is particularly useful for the determination of the primary structure of (carbohydrate) antigens. Finally, electron microscopy reveals ultrastructural details of multimolecular assemblies like viral vaccines.

Circular dichroism spectroscopy - Circular dichroism is a method for monitoring the secondary (far-UV CD) and tertiary (near-UV CD) structure of proteins (97, 98). It utilises circularly polarised light to detect asymmetry in a molecule. The asymmetry, caused by the conformation of proteins, results in differences in absorption of left-handed and right-handed circularly polarised light. In the far-UV region, absorption differences mainly result

from asymmetry in peptide bonds. Thus, the secondary structure is monitored. Relative amounts of α-helix, β-sheet, β-turn and random coil can be calculated. In the near-UV region, absorbance differences are related to relative orientations of absorbing amino acid residues, i.e. tryptophan, tyrosine, phenylalanine and cystine. CD has not only been used to characterise experimental or model antigens (67-69, 99, 100), but also for batch comparison and stability and characterisation studies of common vaccine antigens (Table 6).

Fluorescence spectroscopy - The fluorescence characteristics of proteins are dependent on the close environment of the fluorescent amino acids tryptophan, tyrosine and phenylalanine. Fluorescence spectroscopy is therefore suitable to measure conformational stability of protein antigens. To facilitate the interpretation of fluorescence data, often tryptophan is selectively excited at 295 nm (101). The fluorescence emission maximum and the intensity depend on the exposure of tryptophan to the aqueous phase and the presence of quenching groups (able to dissipate the energy of excited fluorophores, thereby quenching fluorescence). Quenchers can be neighbouring groups present in the protein. Conformational changes may change the proximity of quenching groups, resulting in changes in the fluorescence intensity. By the addition of low molecular weight quenchers like acrylamide, the accessible fraction of the tryptophan residues can be estimated (101). The fluorescence intensity is of course also dependent on the protein concentration and is therefore relatively prone to artefacts. This is not the case if the emission maximum is used as parameter. Upon denaturation tryptophan residues become more exposed to the aqueous phase. The increased polarity of the environment results in a red shift of the fluorescence. An easily measurable difference exists between the maximum of native and denatured protein. By measuring the wavelength shift as a function of a denaturing condition (heat, pH, urea, guanidinium-HCl), the stability of antigens and antigen formulations can be determined (82, 83, 99, 102-104) (Figure 2).
Apart from unfolding processes, covalent changes in the tryptophan residues can be detected, as was demonstrated with tetanus toxoid stored at low pH, a condition that occurs during release from some polymeric microspheres (105).

Fourier transform infrared spectroscopy (FTIR) - The technique uses radiation wavelengths of the infrared region (2500-25000 nm) to excite vibrational levels of molecules. Infrared spectroscopy can be used to identify specific secondary structures. Each secondary structural element (i.e. α-helix, β-sheet, β-turn and random coil) absorbs at specific wavelength regions (98). However, the analysis is not very straightforward, because many proteins contain several of these structural elements. Another disadvantage is the very strong absorbance of water at these wavelengths. Therefore, D_2O is sometimes used as solvent. Futhermore, relatively high protein concentrations are needed. On the other hand, FTIR is the method of choice for analysing the conformation in freeze dried formulations. An advantage of FTIR is that, unlike CD and fluorescence, light scattering usually does not

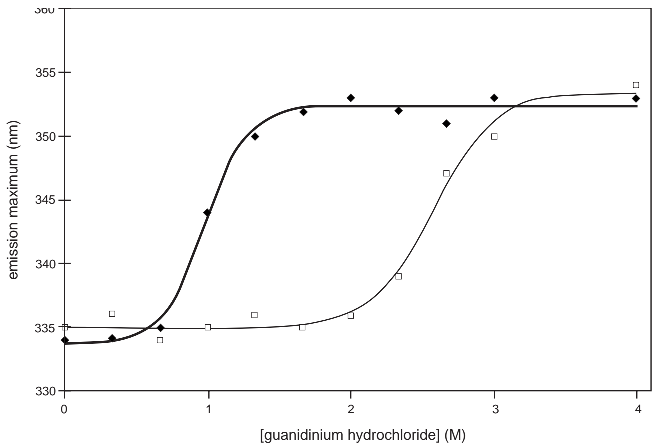

Figure 2. Shift of fluorescence emission maximum of diphtheria toxin (bold line) and toxoid (thin line) as a function of guanidinium concentration. An increasing guanidinium concentration causes a red shift of the emission maximum is an indicator for sensitivity to unfolding. Toxoid is much more insensitive to the denaturing effect of guanidinium. Increased stability may be caused by formation of intramolecular cross-links during formaldehyde inactivation.

interfere, because scatter intensity is proportional to λ^{-4}. FTIR has been used to quantify capsular polysaccharide (106), to reveal the secondary structure of lyophilised tetanus toxoid (107) and for secondary structure determination of hepatitis B surface antigen (108).

Nuclear magnetic resonance (NMR) - NMR uses the magnetic properties of certain nuclei (e.g. ^{1}H, ^{13}C and ^{15}N). By applying a magnetic field and observing the frequency at which they start resonating in an electromagnetic field, structural information is obtained; the resonance frequency is dependent on neighbouring atoms. The technique is therefore very useful for structure elucidation. NMR is becoming a standard technique in the characterisation of polysaccharide vaccines (66, 73, 80, 81, 90, 109-113). Polysaccharide characterisation is not restricted to plain material: NMR is equally suitable for analysing conjugated polysaccharides, because of the much lower signal contribution of the protein carrier.

A validated method has been developed to confirm the identity of bacterial polysaccharides for routine quality control of polyvalent pneumococcal vaccine (113). Besides identity testing, NMR is used to monitor stability of polysaccharide vaccines. Jones and co-workers were able to detect and characterise degradation products in Hib polysaccharide and meningococcal type C-conjugate in accelerated stability studies by use of two-dimensional NMR (114). Although two-dimensional NMR is also suitable for protein research (proteins smaller than 40 kDa), it is not used for routine protein analysis. Reasons for this are that high concentrations of analyte are required and that proteins should be enriched with ^{13}C- and/or ^{15}N-isotopes. Moreover, high-resolution NMR machines are expensive and require expert personnel.

Electron microscopy and dynamic light scattering size determination - Viruses, virus-like particles, vesicles and bacteria can be visualised by electron microscopy. Parameters that can be determined in a semi-quantitative or qualitative way are size, morphology, structural integrity, homogeneity and epitope exposure by labelling with gold particles via antibody-gold conjugates.
In most cases transmission electron microscopy is used, combined with negative staining with metal salts to obtain sufficient electron density. Sample preparation and the high vacuum may induce artefacts. Aggregation may occur or structures may collapse or disintegrate. The presence of crystallising salt or other excipients may shield or blur the object. Preparation techniques less prone to artefacts are available (e.g. freeze fracture techniques), but require special expertise and highly concentrated material. Therefore, size determination of particulate vaccines or antigens by dynamic light scattering is an attractive complementary method for submicron particles (84, 115). This technique makes use of fluctuations in the light scattering intensity as a function of time. Brownian motions, the rate of which is inversely correlated with the particle size, cause these fluctuations. No sample pre-treatment, except sometimes dilution, is needed and small amounts of material are sufficient. On the other hand, (almost) no information about the morphology or integrity of the particles is obtained

The use of in vitro functional tests
An approach to monitor consistency in vaccine production, next to using biochemical or physicochemical models, is the use of *in vitro* and/or *ex vivo* immunological test systems. These systems are based on the use of cells of the immune systems that are kept in culture. Cells can be derived from non-immunised animals or man and even the use of whole blood has been described (42). *In vitro / ex vivo* analysis cannot be considered as replicates of the *in vivo* functioning of the immune system. First, the immune response requires intercellular communication between antigen presenting cells, lymphocytes and non-lymphoid cells such as fibroblast and endothelial cells. This complex communication cannot easily be mimicked *in vitro.* Secondly, some *in vivo* phenomena, such as routes of immunisation and the effect of adjuvants cannot easily be studied *in vitro*. And finally, the antigen dose or load required to induce an effective immune response *in vivo* will be much greater than the antigenic mass required to initiate an immune response *in vitro* (116). So, *in vitro* functional tests are not a replacement for potency tests.
There is another use for *in vitro* functional tests. When animals are vaccinated, the components of their immune response can be analysed *ex vivo*, e.g., for memory induction or bactericidal activity. These tests have the advantage of combining the highly detailed study of the immune response through the use of *in vitro* tests, with the complexities of the *in vivo* immune system (116). Thus, following *in vivo* vaccination, it is possible to study the capacity of lymphocytes from vaccinates to produce antigen-specific antibodies *in vitro* after contact with the antigen. Such *in vitro* analyses can give an accurate determination of

the efficiency with which the immune system would respond to antigen encountered *in vivo.* The disadvantage is that these tests still require the use of laboratory animals.
In the case of demonstration of consistency in production, complex models are not always needed and simplified *in vitro* tests to monitor specific characteristics of the vaccine under study can be highly suitable. In that respect it is essential to know which processes are involved in protection against a particular pathogen. In the case of protection against tetanus and diphtheria, it has been clearly demonstrated that neutralising antibodies constitute the protective mechanism. Studying this response *in vitro* would be ideal, but is difficult. Primary *in vitro* immunisations have been successful when assistance/help was given through the application of cytokine mixtures (117-119). However, the artificial antibody response thus created is not so relevant to vaccine quality control studies (116). An alternative could be the use of pre-processed antigen, wherein antigen-presenting cells (APC) are pulsed with antigen before addition of splenocytes plus fresh antigen (120). By such means, the B-lymphocytes are stimulated directly by the antigen at the same time as the APC-processed antigen stimulates T-lymphocytes. Further improvement can be obtained by co-culture technique, using mononuclear cells from naive donor animals and accessory cells such as fibroblast and endothelial cells.
Another approach is to study cytokine profiles after antigenic stimulation of immune cells *in vitro.* The type and intensity of the immune response are dictated by cytokine production by T helper (Th) cells. Th cells can be classified as either Th1 or Th2 according to the cytokines they secrete (121) and these cytokine profiles can be used to evaluate comparability in immunogenicity. Thus, Leenaars et al. (88) found differences in interferon-γ and interleukin-4 production in supernatants from murine spleen cultures after stimulation with tetanus vaccine batches of varying quality. Instead of using cell cultures, also the use of whole blood has been suggested to monitor cytokine profiles after antigenic stimulation. This system has proven to be effective for the detection of pyrogenic contamination (42) (see also 'test for pyrogens'). Although there is considerable promise for the future application of *in vitro / ex vivo* immunological methods to the analysis of vaccine efficacy, further study will be needed to demonstrate the relevance of these models for routine screening of batches of vaccines.

Validation

In the previous paragraphs several methods have been described that might replace the use of laboratory animals in quality control of vaccines. Whether these methods are accepted in routine testing depends on the outcome of their validation. This makes the demonstration of test validity one of the major cornerstones in the process from test development to implementation.
Unfortunately, however, validation is also a confusing concept, meaning different things to

different people and under different circumstances. As a consequence, validation studies have been performed in the past that ended up in questions rather than in answers. This has resulted in an increased interest in guidelines on how to design and perform a validation study.

Validation comes from the Latin word *valere,* which means 'to have power'. Key-elements in a validation study are the establishment of reliability and relevance of the new method (122). Reliability refers to the level of intra- and interlaboratory test variation and relevance to the usefulness and meaningfulness of the new method for a specific purpose, e.g. the demonstration of potency.

Although a uniform and detailed protocol for all kind of validation studies would make life easier, it is doubtful whether this is the best way forward. New methods might serve different purposes. Thus, *in vitro* tests to assess vaccine potency can be used to demonstrate consistency in production, to show correlation with protection in man or to discriminate between vaccine batches that pass or fail minimum requirements. Each different purpose requires a different validation strategy and this should be reflected in the design of the validation study and the criteria to be met. Sound general principles and the use of common sense, rather than having a set of fixed and dogmatic criteria, offer the possibility to tailor-made a validation study to its particular purpose and to modify it when needed. However, once such a tailor-made design has been agreed upon, it should be followed in every detail (123). Summarising, one could say that the design of a validation study should be a creative and flexible process, while such a study – once designed – should be dictatorial with regard to its performance.

The validation of a new method that is only used as an in-process test or pre-screening test, is relatively simple and can be based on an in house study and the publication of its results in a peer reviewed journal. However, the validation of a new method that is considered for regulatory approval is a tedious and time-consuming process, requiring the involvement of several laboratories. A flow-chart of such a validation study is presented in table 7. Five main stages in the evaluation of a new test method can be identified: test development, pre-validation, validation (involving a formal inter-laboratory study), independent assessment, and progression toward regulatory acceptance. The informal inter-laboratory study carried out at the pre-validation stage involves assessing the inter-laboratory transferability of the method, undertaking any necessary optimisation and standardisation of the protocol and identifying any unexpected problems with the test procedures. The objective of the prevalidation stage is to ensure that any method included in a formal validation study adequately fulfils the criteria defined for inclusion in such a study (124).

In the validation stage, the main objective is to conduct an inter-laboratory blind trial as a basis for evaluating whether one or more tests can be shown to be relevant and reliable for its specific purpose, according to defined performance criteria (122, 125).

The published results of a validation should be considered in detail by one or more independent assessment panels, under the auspices of appropriate national or international

organisations before any regulatory authorities are asked to consider the formal acceptance of the validated method.

Table 7. Stages in the evolution of new tests.[1)]

Stages
Test development (laboratory of origin)
Purpose of the test
Need for the test
Derivation of the method
Case for inclusion in a validation study
Production of a protocol
Prevalidation (informal interlaboratory study)
Optimisation of test protocol
Assessment of interlaboratory transferability
Production of standard operating procedures
Validation (formal interlaboratory study)
Study design
Selection of tests
Selection, distribution and testing of vaccines
Data collection and analysis
Assessment of performance of test
Independent assessment (of study and proposals)
Progression towards regulatory acceptance

1) Balls & Fentem (145)

Concluding remarks

The disadvantages of *in vivo* tests are numerous: they are expensive, inaccurate, slow and ethically questionable. Nevertheless, for many vaccines *in vivo* potency testing is performed on each batch. Opportunities for replacement, refinement and reduction are related with the robustness of the production process, the availability of in-process controls and whether a vaccine is well defined or not defined. In this respect it can be stated that every aluminium salt adsorbed vaccine is not defined, because direct effects on immunogenicity of adsorption of the antigen to aluminium are not measurable. This complicates the development of alternatives for adsorbed (combination) vaccines, which still is a very important category of vaccines. To this end, there is an urgent need for *in vitro* functional tests to determine the potency of these vaccines. This field is starting to emerge and has yet to prove its value. If replacement of challenge tests turns out to be too ambitious, *in vitro* functional tests are attractive refinements. These tests allow the analysis of individual antigens present in combination vaccines, which is by definition not possible when challenge models are used.

With regard to ‘non-functional’ tests, as described in section ‘the fingerprint approach’, the possibilities to characterise vaccines are almost endless. The question here is: which tests

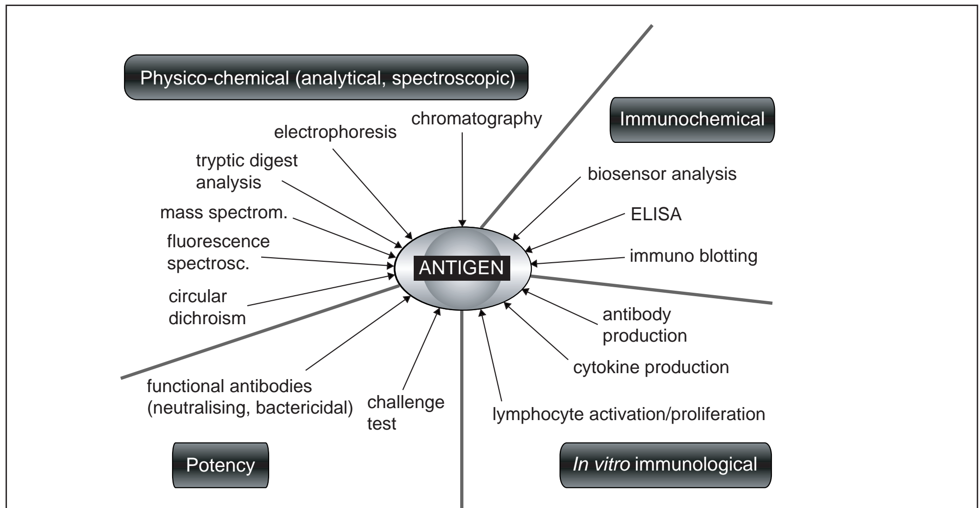

Figure 3. Possibilities for *in vivo* and *in vitro* analysis of protein antigens.

provide relevant information? The choice of the analytical methods should be rational and not based on the available expertise and equipment. It is clear that there is not one single technique that completely characterises an antigen. Always a set of methods should be employed to gain insight into the quality of a vaccine (Figure 3). This transforms quality control and in-process control into a multi-disciplinary activity, requiring collaboration between biochemists, pharmacists, chemists and immunologists.

The final hurdle for the introduction of *in vitro* surrogate tests or the adaptation of *in vivo* tests is their validation and implementation. This phase is laborious, expensive and time consuming. Nevertheless, a number of successful examples is available (Table 8). No routine animal potency tests are currently used for batch release of live bacterial vaccines (oral typhoid, rDNA cholera), live virus vaccines (mumps, measles, rubella, varicella, yellow fever, OPV), hepatitis A and B vaccines, inactivated influenza vaccine, meningococcal and pneumococcal polysaccharides and Hib vaccine. It is anticipated that the list will lengthen, because of:

- the expectation that more new vaccines will be in the 'well-characterised' category, facilitating future *in vitro* comparability studies and the development of in-process controls;
- increased control of critical steps of production processes;
- increased knowledge about immunology, allowing further development of functional tests;
- increased analytical power with regard to antigen characterisation;
- continuous efforts with regard to international harmonisation and validation of test procedures.

Table 8. Successful examples of reduction of animal use in vaccine testing (based on Hendriksen (3) and Dobbelaer (146)).

Vaccine	Old test	New test	Status
Several	Abnormal toxicity / general safety	Deleted after demonstration of production consistency	Regulatory acceptance
Several	Presence of extraneous viruses in virus vaccines on final lots	Presence of extraneous viruses in virus vaccines on seed lots	Regulatory acceptance
Several	Final lot testing	Final bulk testing	Regulatory acceptance
Several	Pyrogenicity in rabbits	LAL	Accepted for Hepatitus A, Typhoid, Yellow fever, Influenza, Rabies, Hib vaccine.
		Whole human blood assay	Experimental phase
Diphtheria toxoid	Potency test in guinea pigs (challenge)	Vero cell assay (immunogenicity)	Validated
Diphtheria toxoid	Residual toxicity in guinea pigs	*In vitro* cytotoxicity	Regulatory acceptance
Tetanus toxoid	Potency test in mice or guinea pigs (challenge)	ToBI/ ELISA	Validated
Whole cell pertussis	Potency test in mice (challenge)	ELISA (147)	To be validated
Rabies vaccine	Potency test in mice (challenge)	Antigenicity (ELISA)	To be validated
HBV	Immunogenicity in mice	Antigenicity (ELISA)	Regulatory acceptance (recombinant products)
HAV	Immunogenicity in mice	Antigenicity (ELISA)	Regulatory acceptance
Yellow fever	LD_{50} in mice	Virus titration	Regulatory acceptance
OPV	Neurovirulence test in monkeys	Transgenic mice	Regulatory acceptance for Sabin type 3
		In vitro mutant analysis (MAPREC)	In proces control for Sabin type 3

Based on (3, 146).

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Chapter 3

Physicochemical and immunochemical techniques predict the quality of diphtheria toxoid vaccines

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Abstract

The most critical step in the production of diphtheria vaccines is the inactivation of the toxin by formaldehyde. Diphtheria toxoid is produced during this inactivation process through partly unknown, chemical modifications of the toxin. Consequently, diphtheria vaccines are difficult to characterise completely and the quality of the toxoids is routinely determined with potency and safety tests. This article describes the possibility of monitoring the quality in diphtheria vaccine production with a selection of physicochemical and immunochemical tests as an alternative to established *in vivo* tests. To this end, diphtheria toxin was treated with increasing formaldehyde concentrations resulting in toxoid products varying in potency and residual toxicity. Differences in the quality of the experimental toxoids were also assessed with physicochemical and immunochemical techniques. The results obtained with several of these analyses, including SDS-PAGE, primary amino group determination, fluorescence spectroscopy, circular dichroism and biosensor analysis, showed a clear correlation with the potency and safety tests. A set of criteria is proposed that a diphtheria toxoid must comply with, i.e. an apparent shift of the B-fragment on SDS-PAGE, a reduction of primary amino groups in a diphtheria molecule, an increased resistance to denaturation, an increased circular dichroism signal in the near-UV region and a reduced binding to selected monoclonal antibodies. In principle, a selected set of *in vitro* analyses can replace the classical *in vivo* tests to evaluate the quality of diphtheria toxoid vaccines, provided that the validity of these tests is demonstrated in extensive validation studies and regulatory acceptance is obtained.

Introduction

Diphtheria toxoid (DTx) is one of the most successful vaccines and has eliminated diphtheria in many countries. It is prepared from diphtheria toxin, which causes the clinical manifestations of the disease in man (1). The toxin is converted by formaldehyde into a non-toxic but still immunogenic diphtheria toxoid. This method has already been described by Ramon and Glenny in the 1920s (2, 3). The classical vaccine is produced via a number of steps: cultivation of *Corynebacterium diphtheriae* and clarification of the toxin-containing medium, followed by concentration and inactivation of the toxin, purification of the toxoid through diafiltration, and adsorption to an aluminium salt. The quality of diphtheria toxoid depends mainly on the detoxification process, in which reaction conditions are very important such as formaldehyde concentration, reaction time and temperature, and composition of the matrix. In many cases, the matrix is not chemically defined and is essentially the same as the culture supernatant, which contains non-specified amino acids, peptides and proteins. Some producers use a defined matrix, which consist of a glycine or a lysine solution. During inactivation, formaldehyde reacts first with amino groups; in a second step, cross-links are formed between the reaction product and several other amino acids (4-6). Thereby, formaldehyde forms intramolecular and intermolecular cross-links. However, the nature of the modifications in the toxoid as well as the location of the modification sites is largely unknown.
The present quality control of diphtheria toxoid is based on an immunogenicity and safety test in animals (7-10). However, most international regulations allow the use of alternative test methods for the quality control of vaccines (11). Importantly, the alternative assay should not pass a product that fails in the routine quality test. Currently, no functional *in vitro* tests exist as an alternative for the potency determination of diphtheria toxoids, because it is very difficult to mimic a complex immune response (12). Another concept for batch release is based on a consistent production process where the vaccine batches predominantly have identical properties (13-15). This is common practise for well-defined biologicals, e.g. hepatitis B vaccine (16). In principle, also the potency of a newly produced toxoid can be predicted, if it can be demonstrated that the new product is indistinguishable from a reference toxoid with a proven potency. Immunochemical and physicochemical techniques are instruments to study vaccine properties, such as identity, size, structure, purity, amino acid modifications and antigenicity. The combination of results can verify that vaccines have identical properties and are consistently produced.
The aim of the present study was to investigate the applicability of physicochemical and immunochemical techniques as quality predictors of diphtheria toxoid. In particular, SDS-PAGE, a primary amino group assay, fluorescence spectroscopy, circular dichroism (CD) spectroscopy and biosensor analysis were used to characterise similarities and differences between a set of experimental diphtheria toxoids. Based on the results, we propose to use a selection of these assays to predict the quality of diphtheria toxoid vaccines.

Materials and Methods

Biochemicals and immunochemicals
Diphtheria toxin-containing culture fluid (clarified and concentrated) was obtained from the production department of the Netherlands Vaccine Institute. Monoclonal anti-diphtheria toxin antibodies Dim 5, Dim 25, Dim 27 and Dim 33 were obtained from the Laboratory for Clinical Vaccines of the NVI. Horse anti-diphtheria toxoid serum, horse anti-diphtheria peroxidase conjugate, diphtheria toxin DTa 79/1 and diphtheria toxoid DTa 93/1 were obtained from the Laboratory for the Control of Biological Products of the NVI.

Preparation of diphtheria toxoids
Before the inactivation of diphtheria toxin, the toxin-containing culture fluid was extensively dialysed (MWCO: 10 – 12 kDa) against PBS (phosphate buffered saline; 0.15 M NaCl, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.2) to remove medium components of low-molecular weight, such as amino acids and peptides. After dialysis, the toxin was filter sterilised (0.22 µm) and the protein concentration was determined to be 3.0 mg/ml by the BCA protein assay (Pierce) (17). The antigenicity was 900 Lf/ml as measured by the Ramon flocculation test (18). For the production of a series of experimental diphtheria toxoids (Table 1), a glycine solution of 2.0 M was added to dialysed toxin to a final concentration of 1, 2, 4, 8, 16, 32, 48, 64, 80 or 128 mM. To start the inactivation reaction, a diluted formaldehyde solution (Merck) of 2.0 M was added to a final concentration equimolar to that of glycine (Merck). A certain amount of PBS was added to the toxoids to obtain a protein concentration of 2.6 mg/ml. The mixtures were incubated for 6 weeks at 35 °C. Diphtheria toxin DTx (2.6 mg/ml) was also incubated for 6 weeks at 35 °C and used as a control. The flocculation titre of the toxin and toxoids was measured and ranged between 750 and 825 Lf/ml. The toxoids were stored at 4 °C prior to analysis.

Table 1. List of experimental toxoids prepared by adding various concentrations formaldehyde and glycine to diphtheria toxin.

Name	Formaldehyde and glycine (mM)
DTx	-
DTd1	1
DTd2	2
DTd3	4
DTd4	8
DTd5	16
DTd6	32
DTd7	48
DTd8	64
DTd9	80
DTd10	128

SDS-PAGE and immunoblotting

SDS-PAGE under reducing conditions and subsequent immunoblot analyses were performed as described (19). For reduction of the disulphide bridges, 2 µg protein was diluted in the 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 for 10 min. The samples of 20 µl were loaded onto 10 % SDS-PAGE gels and electrophoretically separated. SDS-PAGE Molecular weight (broad range; Bio-Rad) were used for calibration. Protein bands were visualised by using Coomassie brilliant blue or via immunoblotting. The gels were scanned and the intensity of protein bands was quantified by using the program Phoretix 1D quantifier (Phoretix International, UK).

For immunoblotting, proteins were transferred to a 0.45-µm nitrocellulose membrane (Bio-Rad) by using a semi-dry electroblot system (Ancos). The protein blots were incubated by shaking for 1 hour with anti-diphtheria toxin antibodies Dim 5 or Dim 25 (1:1000) dissolved in buffer (0.15M NaCl and 10 mM tris(hydroxymethyl) aminomethane), blocked by incubation for 5 min with 0.5% Protifar (Nutricia) diluted in buffer, and treated for 1 hour with horse anti-mouse peroxidase conjugate (1:2000; Organon) dissolved in buffer with 0.5% Protifar. After each incubation step, the blot was thoroughly washed with buffer. Antibody reactive proteins were visualised on the blot by using a substrate solution (10 mM 3,3’, 5,5’-tetramethylbenzidine, 18 mM dioctyl sulphosuccinate, 82.5 mM Na Acetate-buffer, pH 5.5, 25% ethanol and 0.0625% (v/v) hydrogen peroxide (30%)). After the peroxidase-catalysed colour reaction, the blots were washed with water.

TNBS assay

The reaction of formaldehyde with diphtheria toxin results in a reduction of the number of primary amino groups in the molecule. The toxoid samples were dialysed against PBS to remove unreacted glycine. After the dialysis, the protein concentrations and the primary amino group concentration of the toxoids were determined by the BCA protein assay and by a colourimetric assay using 2,4,6-trinitrobenzenesulphonic acid (TNBS) (20), respectively.

Denaturation experiment and fluorescence spectroscopy

The sensitivity of the experimental toxoids to denaturation by guanidine-HCl was studied by fluorescence spectroscopy (21, 22). Toxoid samples of 25 µg/ml were incubated for 2 h with various guanidine-HCl concentrations from 0 - 4 M in steps of 0.2 M. The spectra of the toxoids and toxin were recorded at 25 °C with a Perkin Elmer LS50B fluorescence spectrometer. The excitation wavelength was 295 nm (band width 2.5 nm) and the emission wavelength was measured between 330 and 360 nm (band width 5 nm). For each sample the emission maximum was determined from five averaged scans (corrected for background).

Circular dichroism

Circular dichroism (CD) spectra were recorded at 25 °C with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm) and 1.24-mm slits. Far-UV and near-UV spectra were taken from 260-190 nm (path length 0.2 mm) and from 320-250 nm (path length 10 mm), respectively. The protein concentration was 0.5 mg/ml for far-UV measurements and 1 mg/ml for near-UV measurements. For each measurement six repeated scans (step resolution 1 nm) were averaged and the corresponding buffer spectrum (also six averaged scans) was subtracted. The near-UV CD spectra were smoothed by using a smoothing factor 13. The measured CD signals were converted to molar CD ($\Delta\varepsilon$), based on a mean residual weight of 109.

Biosensor analysis

Biosensor analyses were performed on a Biacore 1000 to measure the affinity of monoclonal antibodies for diphtheria toxin and experimental toxoids. Monoclonal antibodies Dim 5 and Dim 33 are directed against A-fragment of diphtheria toxin, while Dim 25 and Dim 27 can bind the B-fragment. The binding of the experimental toxoids to each antibody was measured. Fc-specific antibodies (Rabbit anti-mouse; Biacore AB) were coupled to a CM5 sensor chip by using an amine coupling kit (Biacore AB) and gave a response of about 5000 resonance units (RU). Subsequently, 500 – 650 RU of anti-diphtheria toxin monoclonal (Dim 5, Dim 25, Dim 27 or Dim 33) diluted in HBS-P buffer (Biacore AB) was bound by the Fc-specific antibody. The experimental toxoids were diluted in HBS-P buffer to a concentration of 300 nM. Finally, the real-time binding and release of the toxoids to the individual monoclonal antibodies were analysed at flow rates of 20 µl/min and for 2.5 and 5 minutes, respectively. For kinetic analysis, the Fc-specific antibodies bound 200 – 400 RU of an individual monoclonal. The binding and release of the toxoid samples were measured for 3 and 5 minutes, respectively. The flow rate was 30 µl/min and the toxin DTx and toxoids DTd4 and DTd5 were diluted to concentrations of 20, 40, 60, 80 and 100 nM. The kinetic data were calculated with BIAevaluation software.

Specific toxicity

The sensitivity of Vero cells to diphtheria toxin provides an opportunity to determine residual toxicity of toxoids (23). Twofold dilution series (between 0.11 – 220 pM) of toxoids were prepared with complete medium 199 (medium 199 (Gibco-BRL) with 10% fetal calf serum, 10000 E/l penicillin and 0.1 mg/l streptomycin), so that each well of the microtitre plate contained a mixture of 100 µl. Subsequently, 50 µl medium with $5{\cdot}10^5$ Vero cells/ml was added to each well. The plates were covered with a plate sealer and incubated for 6 days at 37 °C and 5% CO_2. To determine the viability of the cells (24), 10 µl MTT-solution (5 mg/ml tetrazolium salt in PBS) was added to each well. The plates were incubated for a

further 4 hours in the incubator. Then the medium was removed, 100 µl extraction buffer (100 g/l SDS, 50% (v/v) dimethyl formamide in water and a pH of 4.7, adjusted with acetic acid) was added, the plates were covered again and incubated overnight in the incubator. Finally, the absorbance of the blue-coloured samples was recorded at 570 nm with a plate reader (Bio-kinetics reader EL312e, Bio-tec instruments).

Vaccine preparation

Diphtheria toxoids were diluted to 50 µg/ml (16.6 Lf/ml) in an adsorption mixture (1.5 mg/ml $AlPO_4$ (Adju-Phos®; Brenntag Biosector, Denmark) and 0.15 M NaCl). The samples were mixed by rotating for 24 hours at 4 °C. The adsorption of diphtheria toxoid onto aluminium phosphate was indirectly checked after centrifugation of the samples by a sandwich ELISA on the supernatant, using horse anti-diphtheria toxoid serum and a horse anti-diphtheria peroxidase conjugate (22). The adsorption was between 50 and 80 percent. Before vaccination of mice, the vaccines were diluted in physiological saline solution to a toxoid concentration of 20 µg/ml (ca 6.2 Lf/ml).

Potency determination

Mice were used to determine the potency of experimental diphtheria toxoid vaccines. An amount of 250 µl vaccine (5 µg toxoid) was subcutaneously injected in the groin of each mouse (8 mice per vaccine, NIH, female, weight 10 –14 g). Beside the experimental toxoids, a reference vaccine DTa 93/1 with a potency of 4.15 IU/Lf was used for vaccination. Five groups were injected with 250 µl DTa 93/1 dilutions (containing 18, 9, 4.5, 2.3 and 1.1 Lf/ml, respectively). After 35 days animals were bled and the blood was individually collected in tubes. The blood was incubated for 2 hours at 37 °C and subsequently for 2 hours at 4 °C. The samples were centrifuged for 20 min at 800g. 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. The amount of protecting antibodies was measured by a toxin neutralisation test using Vero cells. Twofold dilution series of individual sera were prepared with complete medium 199 so that each well of the microtitre plate contained 50 µl. Then, 50 µl toxin DTa 79/1 (0.0005 Lf/ml) in complete medium 199 was added to the wells. The plates were incubated for 2 hours at 37 °C. Subsequently, 50 µl complete medium 199 with $5x10^5$ Vero cells/ml was added to each well. The plates were covered with a plate sealer and incubated for 6 days at 37 °C and 5% CO_2. The scores (the number of wells containing living cells) of each vaccine was determined by using the microscope. The living cells form an intact monolayer within 6 days. A reference curve was calculated from the scores of reference vaccine DTa93/1 and used to determine the potency of the experimental toxoids.

Results

SDS-PAGE

Diphtheria toxin normally appears in two structural forms: as a single chain of 58.3 kDa and in the nicked form as two fragments of 21.0 kDa (A-fragment) and 37.3 kDa (B-fragment), which are connected by a disulphide bridge (25, 26). Diphtheria toxin (DTx) and ten experimental toxoids (DTd1 - DTd10; see Table 1) were analysed on an acrylamide gel after reduction of the disulphide bridges. The diphtheria toxin used in this study was almost completely nicked (Figure 1; lane 1). The bands of A-fragment and B-fragment appeared in acrylamide gel at higher apparent masses (27 kDa and 43 kDa, respectively) than expected. A number of differences were found between the toxoids. Firstly, the intensity of the 58-kDa band increased with higher formaldehyde concentrations (lane 1 – 11). Secondly, formaldehyde induced a number of shifts of the B-fragment on the gel, which was verified by immunoblotting and by using a B-fragment specific monoclonal Dim 25 (data not shown). The intensity of the apparent 43-kDa band was reduced with increasing formaldehyde/glycine concentrations, whereas the intensity of an apparent 39-kDa band was increased. The B-fragment was maximally shifted when formaldehyde concentrations above 32 mM were used (lane 7 – 11). A similar effect was observed for the 58-kDa band and the band of the A-fragment, albeit less pronounced. Finally, increasing concentrations of formaldehyde result in broader and more diffuse protein bands.

Primary amino groups

The diphtheria toxin molecule has 40 primary amino groups (25). It is expected that formaldehyde causes a reduction in the number of primary amino groups, because it reacts in the first step with primary amino groups and forms in the second step cross-links with

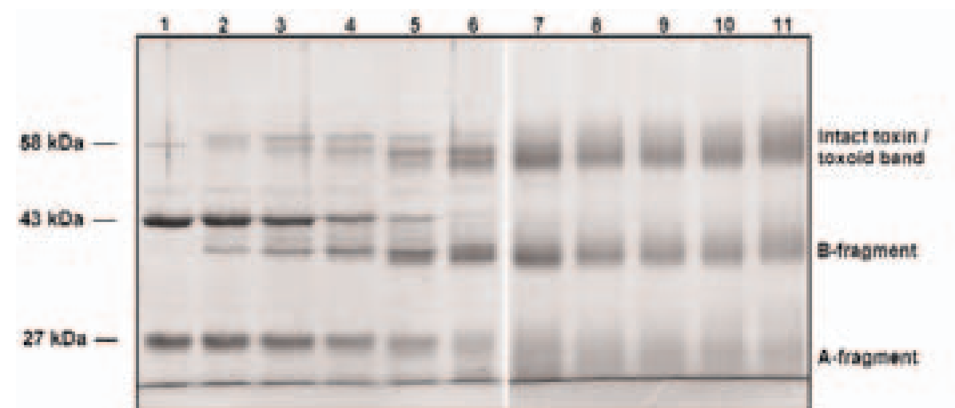

Figure 1. SDS-PAGE of diphtheria toxin DTx (lane 1) and experimental toxoids DTd1 – 10 (lane 2 – 11, respectively). Diphtheria toxoids DTd1 – 10 were prepared with increasing formaldehyde concentrations (see table 1).

other amino acids. The number of primary amino groups has been determined for the experimental toxoids by the TNBS assay. The results are shown in figure 2. With increasing formaldehyde concentration, the number of NH_2-groups was gradually reduced to a minimum level of 40% for DTd6 to DTd10.

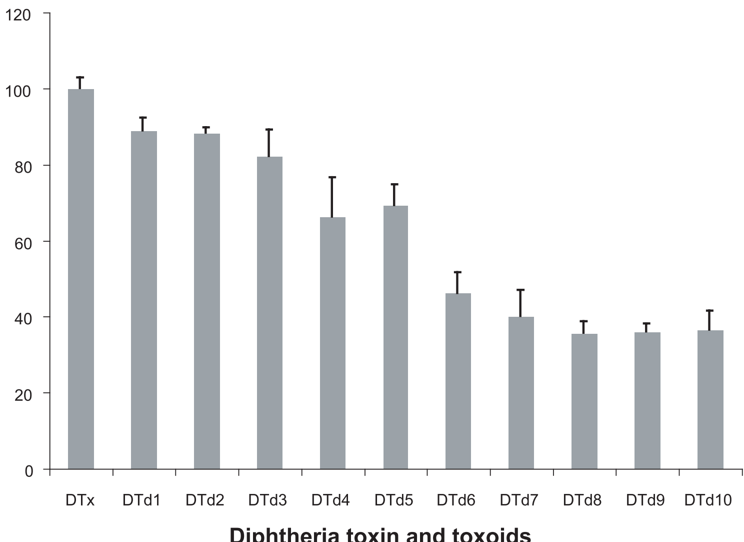

Figure 2. The relative number of primary amino groups in diphtheria toxin and experimental toxoids determined by the TNBS assay and corrected for differences in protein concentration (mean $\pm$ SD; n=6).

Conformational stability

The effect of formaldehyde concentration on the conformational stability was studied by denaturing the toxoids by guanidine-HCl, which was monitored by fluorescence spectroscopy. It has been demonstrated that the toxoid is more resistant to denaturation than the toxin (21, 22). Under physiological conditions, the five tryptophans of diphtheria toxin and toxoids showed an average fluorescence emission maximum around 335 nm. Denaturation causes an increased exposure of Trp residues to the aqueous surroundings, resulting in a shift of the maximal emission to higher wavelengths, in this case to about 353 nm. Figure 3 shows the denaturation curves of diphtheria toxin and the experimental toxoids. Increasing formaldehyde concentrations yielded toxoids that were more resistant to unfolding. However, the resistance to denaturation slightly declined and the denaturation curves became less steep when formaldehyde concentrations were used above 64 mM, e.g. in toxoids DTd9 and DTd10.

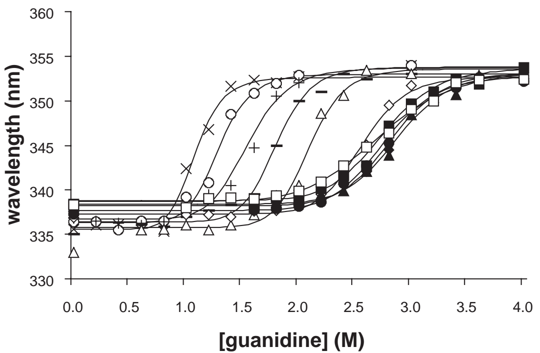

Figure 3. Denaturation of diphtheria toxin DTx (x) and experimental toxoids DTd1 – 10 (○, +, –, △, ◇, ●, ▲, ◆, ■, □, respectively) monitored by fluorescence. Denaturation causes a shift in the emission maximum from 335 nm to 353 nm. The denaturation midpoints are 1.0 M for DTx, and 1.3, 1.5, 1.8, 2.1, 2.5, 2.8, 2.8, 2.8, 2.7 and 2.7 M, DTd1 – DTd10, respectively.

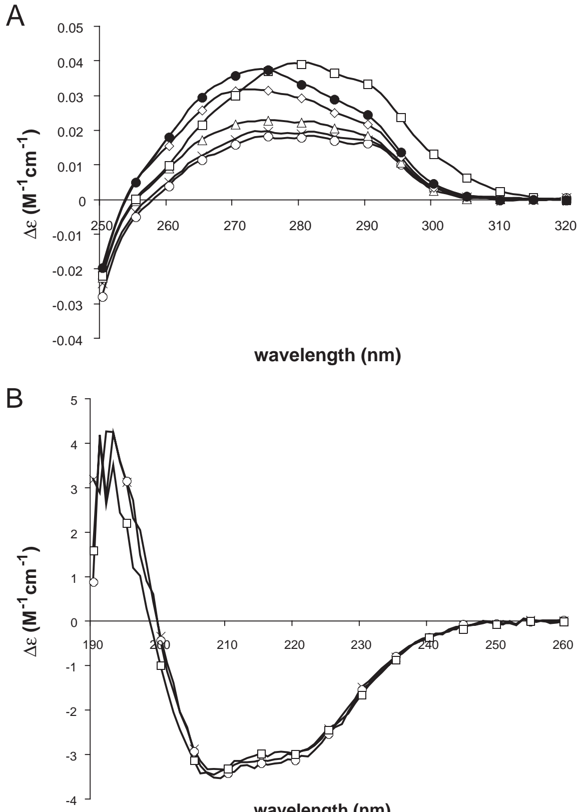

Figure 4. (A) Circular dichroism spectra in the near-UV region obtained for DTx (x) and experimental toxoids DTd1, 4, 5 and 10 (○, △, ◇, ● and □, respectively). (B) Far-UV CD spectra obtained for DTx (x) and experimental toxoids DTd1 and 10. (○ and □, respectively).

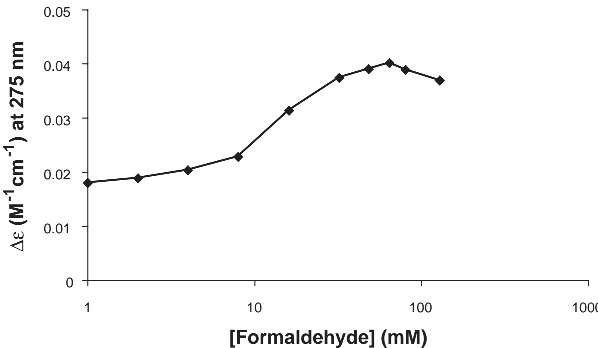

Figure 5. The molar extinction difference (Δε) of the experimental toxoids DTd1 – 10 measured by CD analysis at 275 nm. Diphtheria toxin (DTx) had a molar extinction difference of 0.019.

Circular dichroism

Far-UV and near-UV CD spectra were taken from diphtheria toxin and toxoids to compare their secondary and tertiary structure. Representative spectra are shown in figure 4. Substantial differences between the experimental toxoids were observed in the near-UV CD spectra (Figure 4A). Firstly, a shift in the maximal intensity was observed in the toxoids DTd5 to DTd10 from 275 nm to 280 nm, respectively. Secondly, a sharp transition was observed at a formaldehyde concentration of around 16 mM (Figure 5). These changes indicate that the reaction of diphtheria toxin with formaldehyde induced perturbations of the tertiary structure. In contrast, the far-UV CD spectra of toxin and toxoids were essentially the same, indicating that the secondary structure was unaffected by the detoxification process (Figure 4B).

Biosensor analysis

Four anti-diphtheria toxin monoclonal antibodies (Dim5, Dim 25, Dim 27 and Dim 33) were used in biosensor analysis to measure real-time binding of the experimental toxoids. Each of these monoclonal antibodies has a different specificity. Dim 5 and Dim 33 bind to the A-fragment of diphtheria toxin, whereas Dim 25 and Dim 27 recognise the B-fragment. Competition studies with biosensor analysis have shown that none of the antibodies mutually influence their binding to diphtheria toxin, which means that they recognise a different epitope (unpublished results). In the binding studies, toxin DTx and toxoids DTd1 – DTd10 showed nearly the same binding for monoclonal Dim 5 (Figure 6A). The same holds true for Dim 25 (Figure 6B). This indicates that the epitopes, recognised by these antibodies were largely preserved for each of the toxoids. On the other hand, Dim 27 and Dim 33 (Figure 6C and Figure 6D) showed a decrease of maximal binding for toxoids that were prepared with increasing formaldehyde concentrations. The toxoids DTd6 – DTd10 were not bound at all by these monoclonals. There are at least two explanations for

Table 2. Apparent assocation (k_a) and dissociation constants (k_d) for complexes between monoclonal antibodies and diphtheria toxin (DTx) and toxoids (DTd4 and DTd5).

Antibody	sample[a]	k_a (1/Ms)[b]	k_d (1/s)[b]	K_a (1/M)[b]
Dim 5	DTx	1.6 ± 0.4 x 10^{5}	7.7 ± 0.4 x 10^{-3}	2.1 ± 0.5 x 10^{7}
	DTd4	1.4 ± 0.3 x 10^{5}	5.6 ± 0.7 x 10^{-3}	2.4 ± 0.6 x 10^{7}
	DTd5	1.5 ± 0.4 x 10^{5}	5.5 ± 0.3 x 10^{-3}	2.7 ± 0.8 x 10^{7}
Dim 25	DTx	2.9 ± 0.4 x 10^{5}	1.7 ± 0.1 x 10^{-4}	1.7 ± 0.3 x 10^{9}
	DTd4	3.0 ± 0.5 x 10^{5}	2.0 ± 0.1 x 10^{-4}	1.5 ± 0.3 x 10^{9}
	DTd5	3.3 ± 0.9 x 10^{5}	1.8 ± 0.2 x 10^{-4}	1.8 ± 0.5 x 10^{9}
Dim 27	DTx	1.6 ± 0.1 x 10^{5}	8.2 ± 0.8 x 10^{-4}	2.0 ± 0.6 x 10^{8}
	DTd4	0.9 ± 0.2 x 10^{5}	9.2 ± 0.8 x 10^{-4}	1.0 ± 0.1 x 10^{8}
	DTd5	1.0 ± 0.4 x 10^{5}	10.5 ± 0.5 x 10^{-4}	1.0 ± 0.2 x 10^{8}
Dim 33	DTx	5.7 ± 2.2 x 10^{4}	2.4 ± 1.1 x 10^{-4}	2.4 ± 1.4 x 10^{8}
	DTd4	3.6 ± 2.3 x 10^{4}	4.0 ±2.2 x 10^{-4}	9.0 ± 0.7 x 10^{7}
	DTd5	1.2 ± 1.6 x 10^{4}	7.7 ± 5.5 x 10^{-4}	1.6 ± 0.2 x 10^{7}

[a]Described in table 1.
[b]Mean values ± SD (n = 5).

reduction of the maximal binding; (i) the absolute epitope concentrations were decreased and/or (ii) the epitopes were modified in such a way that they were still recognised by the antibodies, but with a lower affinity. To investigate these options, binding experiments with toxin and toxoids were performed with the four monoclonals to obtain the association and dissociation constants (k_a and k_d, Table 2). There were no large differences between these constants of the toxin DTx and the toxoids DTd4 and DTd5, which have a lower maximal binding to the antibodies Dim 27 and Dim 33. This indicates that the epitope concentration was reduced, but not the affinity for the remaining epitopes.

Cytotoxicity

The residual toxicity was measured with the Vero cell assay. Toxin DTx and toxoids DTd1 – DTd4 showed a measurable cytotoxicity, the extent of which declined with increasing formaldehyde concentration. No residual toxicity was detected for toxoids prepared with formaldehyde concentrations above 16 mM (DTd5 – DTd10; Table 3).

Table 3. The cytotoxic concentration of diphtheriatoxin (DTx) and experimental toxoids (DTd1 – DTd10)

Name	cytotoxic concentration (pM)*
DTx	0.3
DTd1	0.87
DTd2	1.7
DTd3	7
DTd4	55
DTd5 to DT10	> 220

* Determined with the Vero cell assay (see materials & methods).

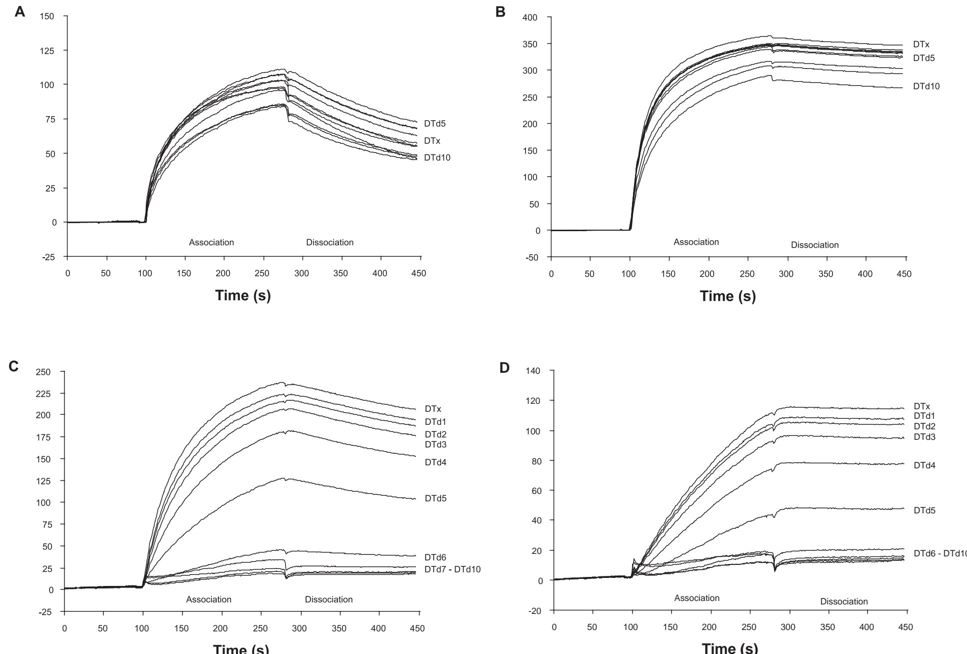

Figure 6. Biosensor analysis of the toxoids with four monoclonal antibodies. The sensorgrams show the binding and dissociation of DTx and DTd1 – DTd10 to (A) the monoclonal antibody Dim 5, (B) Dim 25, (C) Dim 27 and (D) Dim 33.

Potency

The neutralising capacity of sera obtained from mice immunised with an experimental toxoid vaccine was used to determine the potency. Figure 7 shows the results of the potency test. Remarkably, diphtheria toxin (0.7 IU/Lf) had a much lower potency than the reference toxoid DTa 93/1 (4.1 IU/Lf). When increasing the formaldehyde concentration, the potency of the toxoids was gradually enhanced up to a plateau level (ca 11 IU/Lf) for formaldehyde concentrations higher than 32 mM.

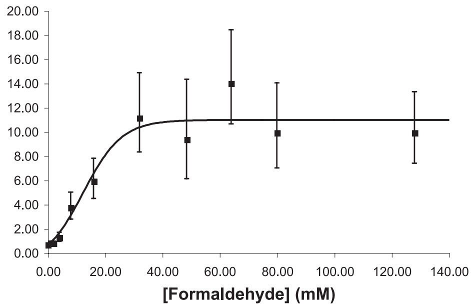

Figure 7. The immunogenicity (IU/Lf) of diphtheria toxoids versus concentration of formaldehyde (mean $\pm$ SE; n=8).

Discussion

Routine quality testing of diphtheria toxoid vaccines is required by the regulatory authorities, including potency and safety tests. These tests rely mainly on the use of animals. The question has been raised if physicochemical and immunochemical tests can be used for the quality control of diphtheria toxoid vaccines (15). These techniques can be used to investigate the characteristics of protein antigens, such as identity, size, structure, purity and amino acid modifications. A set of methods has been selected that may be useful to monitor variation between toxoid batches. The value of each technique has been assessed in this study by using a series of toxoids, prepared with varying formaldehyde and glycine concentrations (Table 1). This study shows that these experimental toxoids varied in immunogenicity and residual toxicity (Figure 7 and Table 3, respectively). Most of the individual analytical methods that were applied could discriminate between these toxoids.

Electrophoretic analysis revealed three types of differences that are caused by the reaction with formaldehyde (Figure 1): (i) a shift of the toxin bands, (ii) change in the ratio of nicked toxoid (21-kDa and 37-kDa fragments) to apparently intact toxoid (58-kDa protein) and (iii) protein bands becoming diffuse. The effects arose successively: first the shifts, then the increased amount of apparently intact toxoid, and finally the broader protein bands. These effects have been attributed to cross-links, which can be formed within the toxin and between amino acids present in the toxoidation medium and the toxin (1, 5, 6, 27). Presumably, the toxin contains some sensitive sites that react very easily with formaldehyde, even at low concentrations. The cross-links that were formed may give the toxin a more compact structure, which would result in a faster migration through the gel (21). An increased amount of intact protein band means that cross-links were formed between the A- and B-fragment. The formation of diffuse protein bands is likely to be a result of heterogeneity of reaction products caused by variable numbers and sites of intramolecular cross-links in the toxoid molecules.

The reaction with formaldehyde reduces the number of primary amino groups per toxin molecule. The number has been determined for all experimental toxoids in a colourimetic assay with TNBS (Figure 2). This reagent reacts with primary amino groups as formaldehyde does. Therefore, the number of primary amino groups per protein molecule is a measure for the extent to which formaldehyde has reacted with the toxin. The primary amino groups were maximally reduced to 40% of the original number in toxoids prepared with high formaldehyde concentrations.

Intramolecular cross-links rigidify the protein conformation, thereby making it more resistant to denaturation (21). Indeed, the more formaldehyde was used for preparation, the more difficult it became to denature the toxoid by guanidine (Figure 3). However, the toxoids DTd9 and DTd10 started earlier to denature at slightly lower guanidine-HCl concentrations and the slopes of these denaturation curves were less steep than those of DTd6 to DTd8. This suggests that less internal cross-links were formed. The relatively high

concentration of glycine in these preparations may be responsible for this effect. If glycine first reacts with formaldehyde, the reaction product can be covalently attached to reactive sites in diphtheria toxin. Thereby, it can inactivate these reactive sites in the toxin that otherwise would react with formaldehyde to form intramolecular cross-links.
The far-UV CD spectra of the toxin DTx and the toxoids DTd1 – DTd10 were the same, indicating that detoxification did not disrupt the secondary structure (Figure 4). An unaffected secondary structure is probably important for the immunogenicity of the toxoids. However, far-UV CD is not suitable for monitoring the detoxification process. In contrast to far-UV CD, differences were found in the tertiary structure as detected by near-UV CD measurements. It demonstrated an increase of the molar extinction differences for toxoids DTd5 – DTd10, suggesting that the aromatic amino acid residues in these toxoids had become less flexible (28). This may be explained by the formation of internal cross-links and is in the line with the results of the denaturation study. An opposite effect is usually observed when protein samples are denatured: this increases the local mobility of amino acid residues, which results in disappearance of the extinction difference (28). Another explanation of the increased extinction difference could be that the aromatic residues were chemically modified by formaldehyde (29), which could result in an intrinsic change of their CD spectrum.
The detoxification of diphtheria toxin has an influence on the antigenicity. This was demonstrated by an increased flocculation time and an underestimation of the toxoid concentration in an ELISA assay compared to the toxin concentration (unpublished data). A difference in antigenicity was also found with monoclonal antibodies as shown in biosensor analysis by measuring the interaction between monoclonal antibodies and toxoids (Figure 6). Some epitopes remained unaffected during the inactivation, while others disappeared. The disappearance of epitopes is most probably caused by chemical modifications of amino acid side chains, although loss of conformational epitopes cannot be excluded on the basis of near-UV CD data. Monoclonals that bind to these sensitive epitopes, such as Dim 27 and Dim 33, can be used to follow the detoxification process. Loss of the epitopes clearly seems to correlate with established potency and absence of toxicity. Dim 5 and Dim 25 bound as strongly to toxin as to the toxoid series. This makes them unsuitable for prediction of vaccine quality, but makes them perfectly suited to determine the antigen concentration. For the determination of the vaccine quality two monoclonals are needed: one that binds with the toxin but not with the toxoid (Dim 27 or Dim 33) and a positive control that binds well to the toxin and the toxoid (Dim 5 or Dim 25). The formation of new, irrelevant epitopes may also be expected. However, monoclonal antibodies directed to such sites were not available for this study.
Whereas the action of formaldehyde had destroyed at least some of the epitopes, the potency of the vaccine had increased about 15-fold for toxoids DTd6 – DTd10 compared to the toxin. Two explanations can be given for this improved immunogenicity. (i) Toxicity of the samples DTd1 to DTd4 impairs antigen-presenting cells and thereby it reduces the

immunogenicity. However, this would be in contrast with the findings of Porro et al, who demonstrated that diphtheria toxin and a non-toxic analogue, CRM197, have the same immunogenicity (30). (ii) An increased stability of toxoid improves the immune response compared to toxin. The internal cross-links in the protein protect against proteolytic degradation, which has an effect on the antigen processing (31) and thereby alters the immune response. For pertussis toxin it has been shown that low formaldehyde concentrations (12.5 mM) slightly improved the immunogenicity, but at higher concentrations (>37.5 mM) the immunogenicity was reduced (32-34). In contrast, in our study diphtheria toxoids prepared with fairly high formaldehyde concentrations (32 – 128 mM) remained very immunogenic. This indicates that formaldehyde did not destroy the important epitopes that are necessary to induce a protective immune response against diphtheria.

The aim of the study was to investigate which *in vitro* techniques are suitable to analyse the quality of diphtheria toxoid vaccines as a possible substitute for the immunogenicity and toxicity tests. Characteristic properties of the physicochemical and immunochemical techniques have to be determined that might predict the quality of vaccine batches. Hence, we defined the following criteria: the B-fragment should be shifted for ≥ 80% from ca 43 kDa to 39 kDa on SDS-PAGE gels; the number of primary amino groups in the toxoids should be reduced to ≤ 40% relative to the number for diphtheria toxin; the denaturation midpoint should be ≥ 2.5 M guanidine; the molar extinction difference at 275 nm determined by CD analysis should be ≥ 0.025 $M^{-1}cm^{-1}$; and the binding of the toxoid by anti-diphtheria antibodies Dim 27 and Dim 33 should be reduced until ≤ 30% of the original intensity. These criteria represent the borderline values, all of which should be met by approved toxoids based on the described parameters.

Based on the immunogenicity and toxicity test, the experimental toxoids could be divided in three categories: (i) toxoids with residual toxicity and low immunogenicity (toxoids DTd1 to DTd4); (ii) borderline products such as toxoid DTd5, having no residual toxicity and inducing a relatively low protecting immune response; (iii) a group of approved toxoids, which are safe and highly immunogenic. These three groups of vaccines were used to establish the minimum criteria the toxoids should fulfil for passing a quality control test. Figure 8 gives a visual impression of the quality of the experimental diphtheria vaccines based on the analytical parameters listed above. If the toxoids have residual toxicity and/or a low immunogenicity, their pentagonal plots are located within the black borderline. Otherwise, the plots cross all the borders and the quality of the toxoids is satisfactory.

In conclusion, the combined application of physicochemical and immunochemical techniques results in a fingerprint of the antigen. The quality of diphtheria toxoid can be predicted on the basis of values determined with the *in vitro* tests used. The reliability of the prediction increases as more *in vitro* methods are applied, because no single analytical technique can completely characterise an antigen. Furthermore, extensive validation studies have to confirm the capability of these tests for quality control. In conclusion, this study

demonstrates that the quality of diphtheria toxoid vaccines can be guaranteed with physicochemical and immunochemical techniques. We expect that similar fingerprint strategies are applicable for other (toxoid) vaccines.

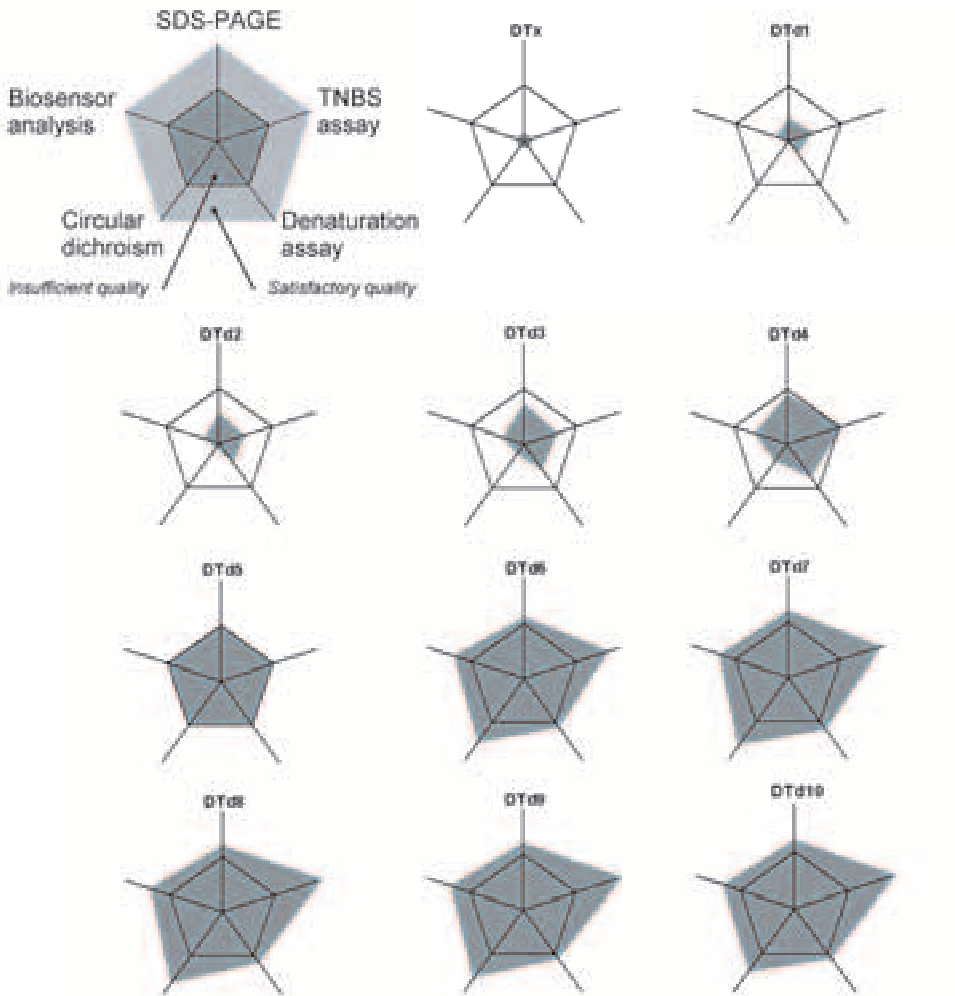

Figure 8. Visual representation of the quality of Diphtheria toxin and experimental toxoids, as characterised by five physicochemical and immunochemical parameters (see text). If grey pentagonal plots are located within the black borderline, the quality of the toxoids is insufficient, e.g. DTx, DTd1 – DTd4. DTd5 is a borderline product. The grey plots of DTd6 – DTd10 cross the black borderlines, indicating satisfactory quality of these toxoids. Scales (from midpoint to limit) are for: SDS-PAGE, the intensity of the apparent 43-kDa band against the 39-kDa band (0% to100%); TNBS assay, the relative amount of primary amino groups in the toxin molecule (100% to 40%); denaturation assay, denaturation midpoint (1.0 M to 3.1 M guanidine); circular dichroism, $\Delta\varepsilon$ at 275 nm (0.018 to 0.043 $M^{-1}cm^{-1}$); biosensor analysis, relative binding intensity to Dim 33 (110 to –26 RU).

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Chapter 4

Quality control of routine, experimental and real-time aged diphtheria toxoids by physicochemical and immunochemical techniques

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Dirk Mekkes, Wim E. Hennink, Gideon Kersten

Abstract

Physicochemical and immunochemical techniques can be utilised to assess the quality of diphtheria toxoid vaccines. In our previous paper (B. Metz et al. Vaccine, 2003. 22: 156-167), we revealed characteristic properties of diphtheria toxoid with SDS-PAGE, primary amino group determination, fluorescence/denaturation, circular dichroism, and biosensor analyses. The *in vitro* analytical tests demonstrated a good correlation with the routine potency and safety tests. However, the analyses were performed with experimental toxoids from one toxin batch. In the present study, we investigated the quality of regular vaccine batches of different manufacturers, the properties of real-time aged products (batches up to 37 years old), and a number of experimental toxoids that were prepared by adapted inactivation procedures, using the above-mentioned analytical techniques. The assays revealed various physicochemical differences between diphtheria toxin and toxoid, i.e. routinely prepared toxoids appeared as a diffuse protein band on SDS-PAGE; had a reduced number of primary amino groups; were more resistant to denaturation; and showed reduced binding to certain monoclonal antibodies as a result of formaldehyde-affected epitopes. The real-time aged diphtheria toxoids showed hardly any differences with the recently prepared products in both the analytical chemical techniques and the conventional potency and safety tests. The analytical assays discriminated between regular diphtheria toxoids and experimental toxoids prepared by methylation, acetylation, or glutaraldehyde treatment. Based on the results, we refined the described physicochemical and immunochemical criteria that a standard diphtheria toxoid have to meet. In conclusion, the analytical data showed a clear correlation with potency and safety of these toxoids. We recommend applying these techniques for quality control of diphtheria toxoid vaccine because of their high accuracy and easy performance as compared to conventional procedures with test animals.

Introduction

The primary concern in the vaccine industry is not the optimisation of product yield or the reduction of manufacturing cost, but the production of human vaccines with consistent high quality (1). To this end, regulatory authorities formulated guidelines to ensure a high level of efficacy and safety. Vaccines are tested in various stages of the production process to comply with the formulated requirements. Unfortunately, animal studies still play a vital role in the testing procedures. However, manufacturers, regulatory authorities and ethical commissions for animal welfare have strong interests in alternative quality testing. Reasons for reduction and replacement of animal tests are their long duration, inaccuracy, high costs and ethical considerations. Currently, a large number of physicochemical, biochemical and cell-based assays are applied for the quality control of vaccines (2-5).

In a previous paper (6), we described five *in vitro* techniques that can be used to monitor the quality of diphtheria toxoid vaccines. The collection of alternative tests consists of SDS-PAGE, primary amino group determination, fluorescence/denaturation assay, circular dichroism, and SPR-biosensor analysis. The assays revealed different vaccine properties, such as identity, size, purity, amino acid modifications, secondary and tertiary structure, and antigenicity. Characteristics of diphtheria toxoids were studied and the essential properties for the quality of the vaccine specified in such a way that they correlated with potency and residual toxicity. Five criteria were formulated that diphtheria toxoid vaccines have to meet for a satisfactory quality (Table 1). However, this study was performed with experimental toxoids prepared from one batch of purified diphtheria toxin that was inactivated in a chemically defined matrix, including formaldehyde and glycine.

The present study focuses on the quality assessment of sixteen diphtheria toxoid batches, including seven routine, six aged and three experimental diphtheria toxoids. The purpose was to investigate whether the physicochemical and immunochemical techniques are generally applicable for quality control. The measurements were used to verify the defined criteria for diphtheria toxoids of desired high quality. We showed that the quality of these toxoids could be assessed by using these techniques.

Table 1. Criteria defined for high-quality diphtheria toxoids.[a)]

Method	Criterion	Borderline
SDS-PAGE	Apparent Shift of B-fragment	≥ 80% from 43 kDa to 39 kDa
TNBS assay	Primary amino groups	≤ 50% relative to DTx
Denaturation assay	Guanidine denaturation midpoint	≥ 2.5 M guanidine
Circular Dichroism	Δ Molar extinction (275nm)	≥ 0.025 M^{-1} cm^{-1}
Surface plasmon resonance	Binding to Dim 33	≤ 30% of DTx intensity

[a)] Criteria defined based on a previous study (6).

Materials and methods

Biochemicals and immunochemicals

Sixteen different diphtheria toxoids were obtained from three different manufacturers (Table 2). These toxoids were classified as regular, aged, and experimental products based on their production date and preparation method. Diphtheria toxin-containing culture fluid (clarified by ultrafiltration and concentrated by diafiltration) was obtained from the production department of the NVI. Monoclonal anti-diphtheria toxin antibodies Dim 5, Dim 25, Dim 27 and Dim 33 were obtained from the Laboratory for Vaccine Preventable Diseases of the National Institute of Public Health and the Environment (RIVM) (6). Horse anti-diphtheria toxoid serum, horse anti-diphtheria peroxidase conjugate, reference diphtheria toxin DT 79/1 and diphtheria toxoid DTa 93/1 were obtained from the Unit Quality Control of the NVI.

Preparation and purification of toxoids

The regular and aged toxoids were prepared according to the validated production method of the individual manufacturers. Because of company policy, the names of the manufacturers remained unspecified. The experimental toxoids DTd14, DTd15 and DTd16 were prepared by a different chemical inactivation method than the standard formaldehyde and glycine treatment: (i) acetylation of lysine residues with acetic acid N-hydroxy succinimide ester, (ii) methylation by formaldehyde and $NaCNBH_3$, and (iii) glutaraldehyde

Table 2. Diphtheria toxoids used in the present study.

Name	Manufacturer	Product
DTd01	NVI	aged (1967)
DTd02	NVI	aged (1970)
DTd03	NVI	aged (1976)
DTd04	NVI	aged (1979)
DTd05	NVI	aged (1982)
DTd06	NVI	aged (1993)
DTd07	A[a)]	regular
DTd08	A	regular
DTd09	A	regular
DTd10	B	regular
DTd11	B	regular
DTd12	B	regular
DTd13	C	regular
DTd14	NVI	experimental[b)]
DTd15	NVI	experimental
DTd16	NVI	experimental

[a)] Manufacturers A, B, and C are anonymous because of company policy.

[b)] The experimental toxoids are prepared by an adapted inactivation procedure (see Materials & Methods for details)..

treatment were applied. At first, diphtheria toxin was extensively dialysed (MWCO: 10 – 12 kDa; Cellucept T3; Membrane Filtration Products, Inc.; USA) against PBS (phosphate buffered saline; 0.15 M NaCl, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.2) to remove medium components of low-molecular weight, such as amino acids and peptides. After dialysis, the toxin was filter sterilised (0.22 μm) and the protein concentration was determined by the BCA protein assay (Pierce, USA) (7). For the acetylation, 10 μl of 0.8 mM acetic acid N-hydroxy succinimide ester (Sigma, NL) dissolved in formamide (Merck) and 500 μl of 1.0 M phosphate buffer, pH 8.5 (988 mM Na_2HPO_4 and 12 mM NaH_2PO_4) was added to 4.5 ml of 3 mg/ml diphtheria toxin. After mixing, the solution was incubated for 24 h. at 37 °C. For the methylation, 400 μl of 1.0 M formaldehyde and 400 μl of 1M $NaCNBH_3$ (Sigma) were added to 4.2 ml of 3 mg/ml diphtheria toxin. The mixture was incubated for 24 h at room temperature. For the inactivation by glutaraldehyde, 200 μl of 2.0 M glutaraldehyde was added to 4.8 ml of 3 mg/ml diphtheria toxin. The solution was incubated for 1 week at 37 °C. Prior to further analyses, all sixteen toxoids were extensively dialysed against PBS (MWCO: 10 – 12 kDa), the protein concentrations were determined by the BCA protein assay, and samples stored at 4 °C.

ELISA for determining toxoid concentration

The diphtheria toxoid concentration (Lf/ml) of each sample was determined by a sandwich ELISA, using horse anti-diphtheria toxoid serum and a horse anti-diphtheria peroxidase conjugate (8).

SDS-PAGE

SDS-PAGE was performed under reducing conditions, essentially as described before (9). Protein samples were prepared by mixing 2 μg toxoid in the sample buffer (60 mM Tris pH 7.2, 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 for 10 min to denature the protein and to reduce the disulphide bridges. The samples were loaded onto 10 % SDS-PAGE gels and the components were electrophoretically separated. Molecular weight reference (broad range; Bio-Rad, USA) was used for calibration. The protein bands were visualised by using Coomassie Brilliant Blue. The gels were scanned and the intensity of the protein bands was quantified by using the program Phoretix 1D quantifier (Phoretix International, UK). Using the intensity profile, the width of each protein band was determined at half intensity.

TNBS assay

The number of primary amino groups in diphtheria toxoids was determined by using a colourimetric assay by using 2,4,6-trinitrobenzenesulphonic acid (TNBS; Fluka, NL) (10). Data obtained from toxoids are presented as relative amount of primary amino groups with regard to diphtheria toxin.

Denaturation of toxoids

Denaturation of the diphtheria toxoids caused by guanidine-HCl (Sigma) was examined by fluorescence as described previously (8, 11). Samples were prepared containing 75 μg/ml toxoid with guanidine-HCl concentrations from 0 to 4 M in steps of 0.25 M. The spectra were recorded with a fluorescence spectrometer (Perkin Elmer LS50B) at an excitation wavelength of 295 nm. Five emission spectra were recorded from 330 to 360 nm, and the emission maximum was determined from the averaged scans. Data are presented as the guanidine-HCl concentration that causes 50% of the total shift of the emission maximum to higher wavelengths (midpoints).

Circular dichroism

Circular dichroism (CD) spectra were recorded at 25 °C with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). Near-UV spectra were taken from 320-250 nm (path length 10 mm). The measured CD signals (ΔA) were converted to molar extinction differences ($\Delta\varepsilon$), according to the equation: $\Delta A = \Delta\varepsilon \cdot c \cdot l$ where c is the amino acid concentration (M) and l is the path length in the cuvette (cm).

Biosensor analysis

SPR biosensor analyses to measure the binding of each toxoid to monoclonal antibodies Dim 5, Dim 25, Dim 27 and Dim 33 were performed on a Biacore 1000 as described earlier (6). Briefly, Fc-specific antibodies (Rabbit anti-mouse; Biacore AB, Sweden) were coupled to a CM5 sensor chip resulting in a response of about 5000 resonance units (RU). Subsequently, an anti-diphtheria toxin monoclonal was captured (between 500-650 RU). Finally, the binding and dissociation of each toxoid (17.5 μg/ml) was measured for 3 and 5 minutes, respectively.

Specific toxicity

Residual toxicity of toxoids was determined by using Vero cells, essentially as previously described (6). Briefly, two equivalent dilution series of each toxoid were prepared in a concentration range between 0.01 Lf/ml (ca. 0.1 μg/ml) and $6 \cdot 10^{-6}$ Lf/ml (ca. 0.05 ng/ml). To one of the two series, horse anti-diphtheria toxin (0.04 IU/ml) was added to neutralise specific toxicity. Subsequently, medium with Vero cells was added to each well. After one week incubation, the viability of the cells was determined by using tetrazolium salt (Sigma) (12).

Vaccine preparation

Sixteen experimental diphtheria vaccine formulations were prepared from the toxoid bulk materials. Therefore, diphtheria toxoids were mixed with aluminium phosphate and sodium chloride to final concentrations of 50 μg/ml toxoid, 1.5 mg/ml $AlPO_4$ (Adju-Phos®; Brenntag Biosector, Denmark) and 0.15 M NaCl.

Potency determination
The potency of the experimental vaccines was determined in a serological test by using mice as earlier described (6). Briefly, eight mice were immunised with 250 μl vaccine containing 20 μg/ml (between 0.2 and 5.1 Lf/ml) toxoid. Vaccine DTa 93/1 with a potency of 4.15 IU/Lf was used as a reference. Thirty-five days after immunisation, animals were bled to obtain individual serum samples. The immunogenicity was measured by toxin neutralisation using Vero cells. A reference curve was calculated from the scores of reference vaccine DTa 93/1 and used to determine the potency of the toxoids.

Results

SDS-PAGE
Diphtheria toxin (DTx) and sixteen toxoids (DTd1 - DTd16) were analysed on an acrylamide gel after reduction of the disulphide bridges (Figure 1). Diphtheria toxin normally appears in two structural forms: as a single chain of 58.3 kDa and in the nicked form as two fragments of 21.0 kDa (A-fragment) and 37.3 kDa (B-fragment), which are interconnected by a disulphide bridge (13). In the present study, diphtheria toxin appeared almost completely in the nicked form as two fragments: (i) A-fragment of 21.0 kDa (observed as 24 kDa) and (ii) B-fragment of 37.3 kDa (observed as 42 kDa).
The diphtheria toxoids were observed as diffuse protein bands as a result of formaldehyde treatment (6). Variations within the diphtheria toxoids were seen by SDS-PAGE. The oldest diphtheria toxoids (DTd1 – DTd6) appeared as partially nicked products, except for DTd4. Protein bands were seen of apparently intact toxoid (ca. 58 kDa), A-fragment (ca. 21 kDa) and B-fragment (ca. 38 kDa). The toxoid DTd4 appeared as intact toxoid (ca. 58 kDa) and as cross-linked dimer (ca. 120 kDa). Recently produced batches (DTd7 – DTd12) gave only one band of the intact toxoid. Toxoid DTd13 was partially nicked with a similar appearance as the aged toxoids (DTd1 – DTd6). Toxoids that were prepared by alternative inactivation

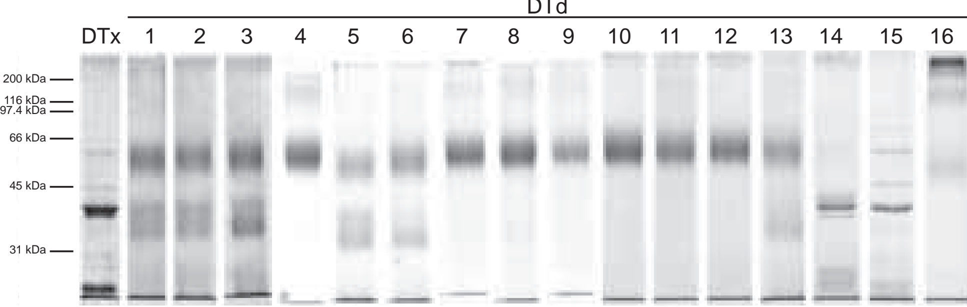

Figure 1. SDS-PAGE of diphtheria toxin and sixteen toxoids.

procedures (DTd14 – DTd16) showed different patterns on the acrylamide gel. Acetylation of lysine residues in DTd14 resulted in slightly higher masses of the A-fragment and B-fragment. Moreover, the dimethylation of lysine residues revealed in DTd15 shifted protein bands. The glutaraldehyde treatment of diphtheria toxin resulted in monomeric, dimeric and multimeric structures. Moreover, the monomeric structure of glutaraldehyde-treated diphtheria toxin appeared as a diffuse protein band with an apparent lower mass than the regular and real-time aged toxoids (DTd1 – DTd13).

Primary amino groups

Formaldehyde treatment of diphtheria toxin causes a reduction in the number of primary amino groups (6). The decrease has been determined for all toxoids by the TNBS assay. The results are shown in Figure 2. Compared to diphtheria toxin, the number of amines in these toxoids was reduced to a level between 10% - 70% of the original value. Regular toxoid batches (DTd7 – DTd 13) had comparable numbers of primary amino groups (40% compared to DTx). In contrast, the aged products had in general a higher number of NH_2-groups (50% -70%) except for toxoid DTd4 (29%). Possibly, new amino groups are formed in these toxoids by the degradation of the protein backbone. This phenomena is sometimes observed in aged biologicals (14). The toxoids DTd7 (23%), DTd15 (11%) and DTd16 (21%) had a remarkably low number of amino groups.

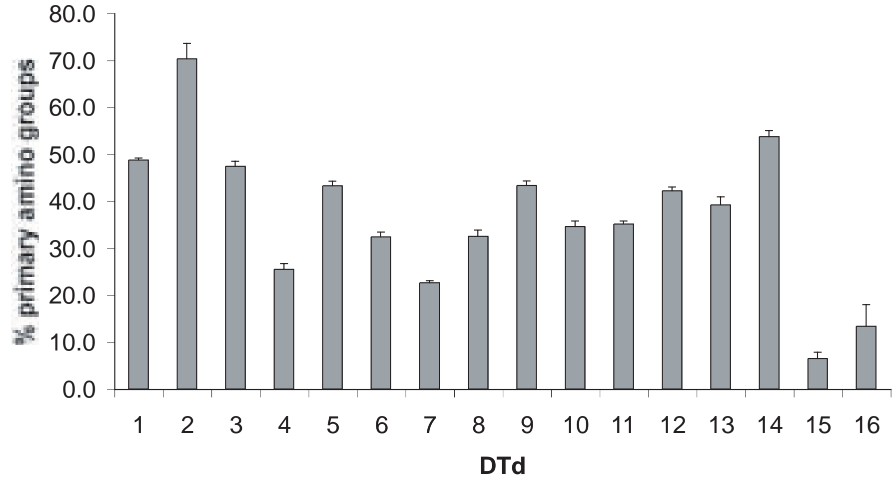

Figure 2. The relative amount of primary amino groups in sixteen diphtheria toxoids. (Diphtheria toxin = 100%).

Conformational stability

Diphtheria toxoids are more resistant to denaturation by guanidine-HCl than the toxin (8, 11). The guanidine-HCl induced denaturation of diphtheria toxin and sixteen toxoids was studied by measuring the shift of the tryptophan fluorescence emission. The denaturation midpoints are shown in table 3. The regular and aged toxoids (DTd1 – DTd13) denatured at high guanidine-HCl concentrations, i.e. above 2.5 M. In general, the denaturing guanidine-HCl concentrations for the aged toxoids were slightly lower than the more recently produced batches. The experimental toxoids (DTd14 – DTd16) denatured at low guanidine-HCl concentrations, comparable to diphtheria toxin. Remarkably, it was observed that absolute fluorescence intensity of the oldest toxoids was lower than the more recently produced toxoids, probably due to oxidation of tryptophan (15).

Table 3. Characterisation of diphtheria toxoids.[a)]

Toxoid	Purity	conformational stability [b)]	Biosensor analyses[c)]				Cytotoxic concentration
	(Lf/μg)	(M)	Dim5	Dim25	Dim27	Dim33	(Lf/ml)
DTx	0.3	1.5	100	100	100	100	$<6 \cdot 10^{-6}$
DTd1	0,13	2.9	122	76	36	9	>0.01
DTd2	0,12	2.6	146	82	37	8	>0.01
DTd3	0,24	2.9	186	88	51	22	>0.01
DTd4	0,16	3.1	79	84	12	3	>0.01
DTd5	0,24	3.0	142	82	66	14	>0.01
DTd6	0,17	3.2	127	86	39	9	>0.01
DTd7	0,16	3.6	69	85	5	3	>0.01
DTd8	0,12	3.5	72	76	3	3	>0.01
DTd9	0,11	3.3	54	71	2	17	>0.01
DTd10	0,43	3.7	73	61	17	-11	>0.01
DTd11	0,30	3.7	74	54	16	7	>0.01
DTd12	0,32	3.6	99	68	22	8	>0.01
DTd13	0,19	3.5	143	84	63	8	>0.01
DTd14	0,12	1.8	81	28	50	3	0.002
DTd15	0,04	2.0	6	-1[d)]	-2	-7	>0.01
DTd16	0,06	2.0	0	-1	-5	-14	>0.01

[a)] Several characteristic properties of toxoids were determined, such as conformational stability, antibody binding (biosensor analysis), potency and cytotoxicity (see Materials andMethods for details).

[b)] Guanidine-HCl concentration for 50% denaturation of the toxoid (midpoints) .

[c)] Percentage of binding of toxoids (DTd1 – DTd16) to individual monoclonals calculated with respect to diphtheria toxin (DTx).

[d)] A negative response was observed, indicating that no binding of toxoid and dissociation of the monoclonal antibody occurred.

Circular dichroism

Near-UV CD spectroscopy has been shown to be useful for monitoring the tertiary structure of experimental toxoids (6). Also in the present study, near-UV CD spectra showed large differences between the toxoids. Several near-UV CD spectra are given in Figure 3. Most routine and aged toxoids showed a lower ellipsicity at 275 nm than observed for diphtheria toxin. Those spectra were comparable to that of DTd1 (Figure 3). However, recently prepared toxoids (DTd 8 and DTd9) showed an increased intensity at 275 nm. The experimental toxoids (DTd14 and DTd15) demonstrated an almost similar spectrum as diphtheria toxin, indicating that neither actelylation nor methylation of lysine residues changes the tertiary structure (Figure 3). In contrast, glutaraldehyde treatment drastically changed the structure (see Figure 3, DTd16).

Biosensor analysis

The binding of diphtheria toxin and toxoids to the monoclonal antibodies Dim 5, Dim 25, Dim 27 and Dim 33 was measured. Dim 5 binds better to six toxoid batches as compared to toxin (Table 3), whereas the other batches showed reduced binding to this monoclonal. Reduced binding to monoclonal Dim 25 was detected for diphtheria toxoids DTd1 – DTd13, as compared to toxin DTx. This indicates that the epitope for Dim 25 was at least partly in the regular and aged toxoids.

The epitopes recognised by Dim 5 and Dim 25 were completely destroyed by methylation of lysine residues or by the glutaraldehyde treatment, as was found for toxoids DTd15 and DTd16. In a previous study, we observed that toxoids completely inactivated by formaldehyde and glycine do not bind to Dim 27 and Dim 33 (6). However, toxoids

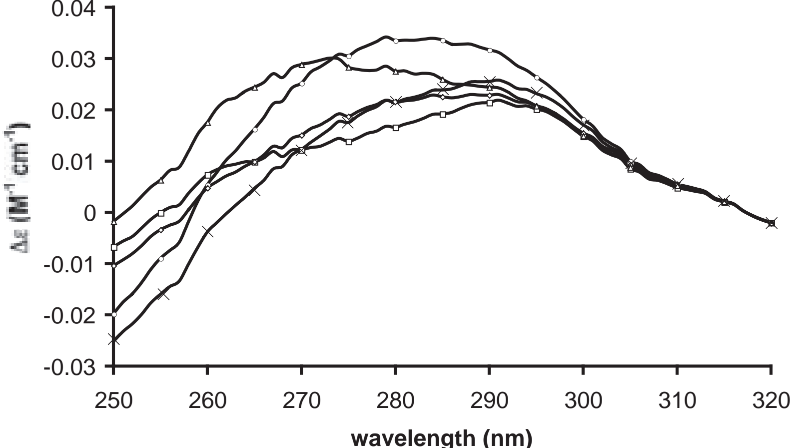

Figure 3. Representative near-UV CD spectra of diphtheria toxin DTx (x) and toxoids DTd1 (□), DTd9 (○), DTd15 (◇), and DTd16 (△).

analysed in the current study varied widely in their ability to bind to Dim 27. Substantial binding was detected for the toxoids DTd1 – DTd6, DTd13 and DTd14, whereas little binding was measured for toxoids DTd10 – DTd12 (Table 3). For toxoids DTd7 – DTd9, DTd15 and DTd 16, no binding occurred to Dim 27. On the other hand, biosensor analyses showed that none of the toxoids bind to Dim 33 (Table 3).

Cytotoxicity
The residual toxicity of each toxoid was measured by using Vero cells (Table 3). The toxoids DTd1 – DTd13, DTd15 and DTd16 showed no cytotoxicity in the concentration range tested. Only, toxoid DTd14 demonstrated residual toxicity, although declined as compared to diphtheria toxin. The cytotoxicity could be neutralised by horse anti-diphtheria toxin serum indicating that the toxicity is specific for diphtheria toxin.

Potency
The potency was assessed for each individual toxoid in the toxin neutralisation test. Therefore, the toxin neutralising capacity of sera obtained from immunised mice was determined by using Vero cells. Figure 4 shows the results of the potency test. The potency values varied between the toxoids (0 and 8 IU/Lf). Toxoids DTd4 and DTd9 gave the highest potency values, whereas toxoids DTd10 – DTd12 had a significantly lower potency than the other regular and aged toxoids (DTd1 – DTd9). The experimental toxoids did not reveal any neutralising activity (DTd14– DTd16).

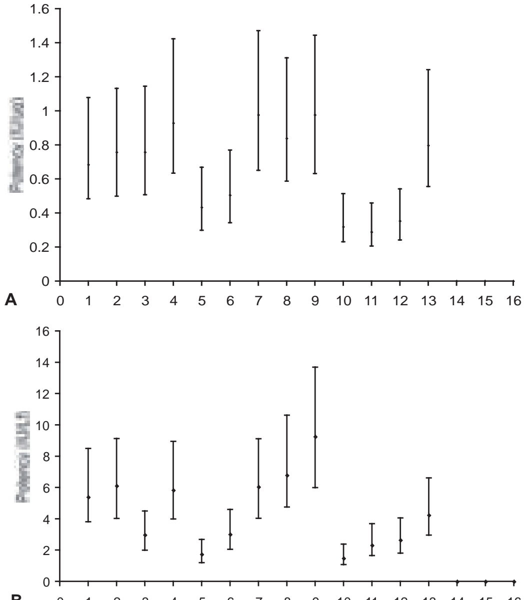

Figure 4. The potency of diphtheria toxoids. The data is presented as (A) IU/μg and (B) IU/Lf (mean ± S.E.; n = 8).

Discussion

In a previous study with experimental diphtheria toxoids, several *in vitro* analytical tests demonstrated correlation with the routine potency and safety tests (6). Currently, a number of regular, real-time aged and other experimental diphtheria toxoids were analysed using the same set of analytical techniques, including SDS-PAGE, primary amino group determination, fluorescence/denaturation assay, circular dichroism, and biosensor analysis. The purpose was to investigate whether the *in vitro* techniques are generally applicable for quality control of diphtheria toxoids. The potency and safety tests have shown that regular and aged toxoids (DTd1 – DTd13) are of reasonable quality, except for toxoids DTd5 and DTd10 which are borderline (Table 3 and Figure 4). The potency of a diphtheria toxoid vaccine must be at least 2 IU/Lf, according to the regulatory authorities (16). In contrast, the experimental diphtheria toxoids (DTd14 – DTd16) did not induce a protecting immune response. Furthermore, toxoid DTd14 revealed residual toxicity. In conclusion, the quality of thirteen routine and aged diphtheria toxoids is satisfactory and three experimental toxoids were inferior in quality. Comparable results were generally found with the *in vitro* analytical tests, which are individually discussed.

With SDS-PAGE, differences were observed between the tested diphtheria toxoids. The routine and aged toxoids (DTd1 – DTd13) showed one or three diffuse protein bands. Probably, the structural form of diphtheria toxin prior to the detoxification by formaldehyde is the determining factor for the number of protein bands. Diphtheria toxin in the intact form gives only one protein band after the formaldehyde treatment, whereas in the nicked form it gives three bands. In our previous study (6), we showed that the inactivation of diphtheria toxin into toxoid causes three distinct changes as is visualised on the acrylamide gel: (i) a shift of the protein bands; (ii) a change in the ratio of nicked fragments (21 and 37 kDa) to apparently intact protein (58 kDa); and (iii) bands becoming diffuse. Especially, the shift of the B-fragment from ca. 43 to 39 kDa was used as an indicative criterion for the quality of diphtheria toxoid. This phenomenon can still be used to assess the quality of the nicked toxoids, e.g. DTd1, DTd3, DTd4, DTd5, DTd6 and DTd13. However for toxoids prepared from intact toxin, the shift of the B-fragment cannot be used as a decisive factor for the quality, because the B-fragment will not be observed in these toxoids. Therefore, we would suggest using the third aspect, which occurs in both types of toxoids: protein bands becoming diffuse. The change in width of the protein band can be determined by measuring the width of the toxin band prior to the detoxification and the width of the toxoid band after the formaldehyde treatment. The width of the protein band at 58 kDa was at least 3 times larger than the original toxoid. For this purpose, the electrophoretic analysis under non-reducing conditions is most suitable. Furthermore, SDS-PAGE also revealed unambiguous differences between the formaldehyde-treated toxoid and the other chemically treated diphtheria toxins (DTd14 – DTd16).

The TNBS assay demonstrated substantial reduction of primary amino groups in all sixteen

toxoids. The number varied between 10 - 70% of the original value (Figure 2).
According to a previously defined criterion, the number of primary amino groups in diphtheria toxoid of sufficient quality is reduced by at least 50% as compared to diphtheria toxin. The aged toxoid DTd2 did not meet this requirement, although the toxoid passed the standard potency and safety tests. Chemical modifications, such as acetylation, methylation or glutaraldehyde attachments resulted in a reduction of primary amino groups. However, the quality of the toxoids DTd14 – DTd16 was very poor, because these vaccines did not induce a toxin-neutralising potency. Thus, the colourimetric assay does not discriminate between the used reagents for inactivation, such as the acetic acid ester, formaldehyde and $NaCNBH_3$, glutaraldehyde, or formaldehyde and glycine. For quality control purposes, this is not necessarily a drawback, because the alternative inactivation procedures are not routinely used.
Diphtheria toxoids are more resistant to denaturation than diphtheria toxin (6, 8, 11). The denaturation of the sixteen toxoids with guanidine-HCl was studied. The regular and aged toxoids DTd1 – DTd13 satisfy the earlier formulated criterion by denaturing at a guanidine-HCl concentration above the 2.5 M. The large differences in guanidine-HCl concentrations (2.6-3.7 M) indicate non-uniform intramolecuclar cross-linking. Old products tend to be less stable than the more recently produced toxoids. In contrast to the routine products, the experimental toxoids DTd14 – DTd16 denatured below a concentration of 2.5 M guanidine-HCl, and do not satisfy the earlier formulated criterion.
Using near-UV CD analyses, we demonstrated in our previous study that formaldehyde treatment affected the tertiary but not the secondary structure of diphtheria toxin (6). Increased molar extinction differences at 275 nm were observed in the experimental toxoids. Near-UV CD measurements performed on the sixteen diphtheria toxoids revealed again changes in the tertiary structure. However, the molar extinction difference was not necessarily higher. On the contrary, most regular and aged toxoids, e.g. DTd1 and DTd12 showed lower near-UV CD signals. Only the most recently produced toxoids, DTd8 and DTd9, gave an increase in the ellipsicity differences. Possibly, small variations in the composition of the matrix for the inactivation, e.g. the concentration of formaldehyde, glycine or medium components, may cause a significant change near-UV CD spectra. In conclusion, the near-UV CD analysis may be indicative for determining consistency in production, but further studies are necessary to demonstrate that the technique is suitable for monitoring the product quality.
The detoxification process changes the antigenicity of diphtheria toxin. The formaldehyde treatment resulted in the disappearance of certain epitopes while others remain unaffected. Monoclonals Dim 5 and Dim 25 bind strongly to diphtheria toxin as well as toxoid, whereas monoclonals Dim 27 and Dim 33 only bind to diphtheria toxin. In the previous study, we demonstrated that a combination of both types of antibodies is perfectly suited to monitor the inactivation process: (i) monoclonals Dim 5 or Dim 25 to determine the identity and concentration of the toxin or toxoid, and (ii) monoclonals Dim 27 or Dim 33 indicate that

the inactivation has occurred (6). However, a remarkable property was revealed in this study for monoclonal Dim 5. The antibody preferentially binds to diphtheria toxoids prepared from nicked toxin as compared to toxoids from intact toxin (Table 3). Probably, the epitope for monoclonal Dim 5 is easier accessible as a result of the nicking. This makes them less suitable for determination the concentration of toxoids. Furthermore, the epitope of the monoclonal Dim 27 was not always completely destroyed in the tested toxoids. Especially, substantial binding was observed for the aged products DTd1 – DTd6. The present study also revealed that the epitopes for Dim 5 and Dim 25 are destroyed in the experimental toxoids DTd14 – DTd16. So, antibodies could discriminate between the inactivation by formaldehyde and other chemicals, such as acetic acid ester, formaldehyde plus $NaCNBH_3$, and glutaraldehyde. Based on all these results, the use of monoclonals Dim 25 and Dim 33 is preferred for quality control of regular toxoids.

The experiments using the above-described techniques revealed unequivocally differences between diphtheria toxin and the tested diphtheria toxoids. In our previous study, we defined five criteria that are achieved by diphtheria toxoids of high quality (Table 1). The present study shows that three criteria remain unchanged for the regular toxoids, i.e. at least 50% reduction of primary amino groups relative to diphtheria toxin; denaturation at guanidine-HCl concentrations above the 2.5 M; and maximal binding of 30% to monoclonal antibody Dim 33 compared to diphtheria toxin. Although SDS-PAGE is indicative for the quality, the previously defined criterion has to be adapted. We would suggest using the change in thickness of the protein band as decisive parameter. The width of the protein band of a prepared diphtheria toxoid is at least 3 times larger than of the original toxin. Near-UV CD measurements revealed that most of the regular and aged diphtheria toxoids do not meet the criterion (Table 1). Detailed research is necessary to determine whether and how the CD technique can be indicative for the quality of routinely produced diphtheria toxoids.
Figure 5 gives a visual impression of the quality of the regular, aged and experimental toxoids based on the results of SDS-PAGE, TNBS assay, denaturation assay and the biosensor analyses. The *in vitro* analytical assays and the potency and safety tests give similar results about the quality of diphtheria toxoids, except for DTd2. In conclusion, the fingerprinting approach (3) by using several *in vitro* techniques is successful to predict the quality of diphtheria toxoid vaccines.

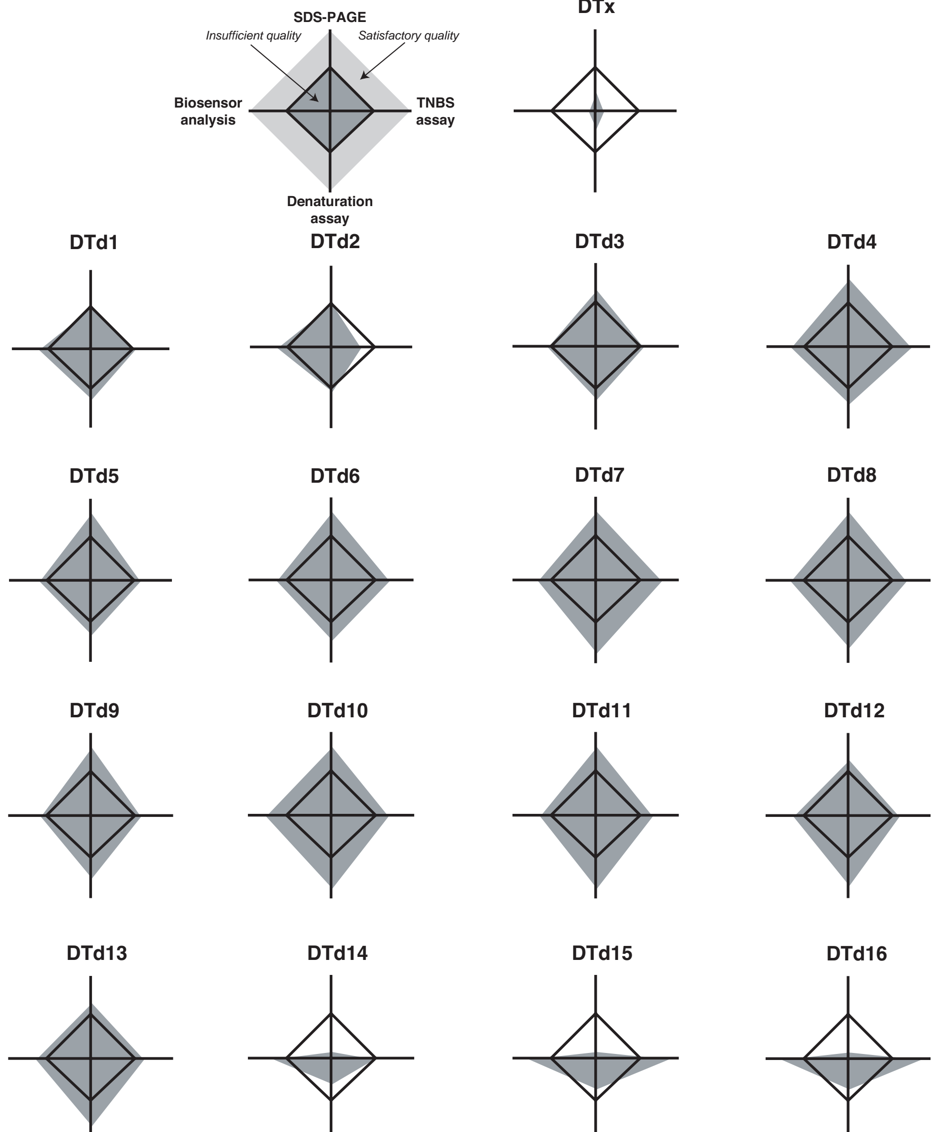

Figure 5. Visual representation of the quality of diphtheria toxin and toxoids. The quality was based on the four criteria adapted from previous our study (6). Toxoids have a satisfactory quality if the grey plot completely crosses the black borderline. Scales (from centre to limit) are for: SDS-PAGE, the width of the toxoid band at 58 kDa divided by the toxin band at 58 kDa (0 – 5 times); TNBS assay, the relative amount of primary amino groups in the toxin molecule (100% to 0%); denaturation assay, denaturation midpoint (1.0 M to 3.9 M guanidine); biosensor analysis, relative binding intensity of toxoid to Dim 33 in relation to toxin (100% to –40%).

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Chapter 5

Identification of formaldehyde-induced modifications in proteins: reactions with model peptides

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Abstract

Formaldehyde is a well-known cross-linking agent to inactivate, stabilise or immobilise proteins. The purpose of this study was to map the chemical modifications occurring on each natural amino acid residue caused by formaldehyde. Therefore, model peptides were treated with excess formaldehyde and the reaction products were analysed by liquid chromatography-mass spectrometry. Formaldehyde was shown to react with the amino group of the N-terminal amino acid residue and the side-chains of arginine, cysteine, histidine, and lysine residues. Depending on the peptide sequence, methylol groups, Schiff-bases and methylene bridges were formed. To study intermolecular cross-linking in more detail, cyanoborohydride or glycine was added to the reaction solution. The use of cyanoborohydride could easily distinguish between peptides containing a Schiff-base or a methylene bridge. Formaldehyde and glycine formed a Schiff-base adduct, which was rapidly attached to primary N-terminal amino groups, arginine and tyrosine residues, and to a lesser degree, asparagine, glutamine, histidine, and tryptophan residues. Unexpected modifications were found in peptides containing a free N-terminal amino group or an arginine residue. Formaldehyde-glycine adducts reacted with the N-terminus via two steps: the N-terminus formed an imidazolidinone, and then the glycine was attached via a methylene bridge. Two covalent modifications occurred on an arginine-containing peptide: (i) the attachment of one glycine molecule to the arginine residue via two methylene bridges, and (ii) the coupling of two glycine molecules via four methylene bridges. Remarkably, formaldehyde did not generate intermolecular cross-links between two primary amino groups. In conclusion, the use of model peptides enabled us to determine the reactivity of each particular cross-link reaction as a function of the reaction conditions and to identify newly discovered reaction products after incubation with formaldehyde.

Introduction

Aldehydes, such as formaldehyde and glutaraldehyde are widely employed reagents in the biochemical, biomedical and pharmaceutical field. Formaldehyde, for example, is applied to inactivate toxins and viruses for the production of vaccines, such as diphtheria and tetanus toxoid, hepatitis A, anthrax, inactivated polio vaccine, and to stabilise recombinant pertussis toxin (1-4). The vaccine quality depends to a considerable extent on the chemical modifications caused by the formaldehyde treatment (1, 5, 6). Formaldehyde is also used for isotope-labelling of proteins (7-9), for studying protein-protein interactions, e.g. histone organisation in nucleosomes (10-12) and for fixation of cells and tissues (13). Glutaraldehyde is utilised for preparation of bioprotheses such as heart valves and vascular grafts (14-16) and for conjugation of enzymes to carrier systems (17). These examples demonstrate the wide range of roles of aldehydes in the biomedical field. Beside the advantage of using aldehydes for diverse applications, they can also destroy important sites of proteins, such as crucial epitopes or active sites in enzymes.

Several decades ago, extensive model studies have been performed on reactions of formaldehyde with mixtures of amino acids and derivatives to determine which amino acids can cross-link (18-21). It was demonstrated that formaldehyde reacts first with amino and thiol groups of amino acids and forms methylol derivatives. In case of primary amino groups, the methylol groups partially undergo condensation to an imine, also called a Schiff-base (Scheme 1). Subsequently, the imine can cross-link with glutamine, asparagine,

$$[1]\quad \text{Protein—}NH_2 + HCHO \rightleftharpoons \text{Protein—}NH\text{—}CH_2OH \quad (\Delta m=+30)$$

$$[2]\quad \text{Protein—}NH\text{—}CH_2OH \ (\Delta m=+30) \rightleftharpoons \text{Protein—}N{=}CH_2 \ (\Delta m=+12) + H_2O$$

$$[3]\quad \text{Protein—}N{=}CH_2 + \text{Protein-phenol (Tyr)} \longrightarrow \text{Protein—}NH\text{—}CH_2\text{—(Tyr)—Protein}$$

Scheme 1. The reaction of formaldehyde with proteins. The reaction starts with the formation of methylol adducts on amino groups [1]. The methylol adducts of primary amino groups are partially dehydrated, yielding labile Schiff-bases [2], which can form cross-links with several amino acid residues, e.g. with tyrosine [3].

tryptophan, histidine, arginine, cysteine and tyrosine residues. Some of the chemical structures of the proposed adducts have been elucidated by NMR (22). This knowledge, however, is not sufficient to predict all possible modifications in proteins, which are induced by formaldehyde. Moreover, the formation of modifications is influenced by various factors; such as the rate of a particular cross-link reaction, the position and local environment of each reactive amino acid in the protein, the pH, the components present in the reaction solution, and the reactant concentrations. Importantly, the nature of all possible chemical modifications in proteins caused by formaldehyde has not been fully elucidated yet. This is in part due to the low resolution and sensitivity of the analytical methods available at the time the above studies were performed (18-21). However, the current availability of tandem HPLC-mass spectrometry allows gaining more detailed insight into the chemistry of protein-formaldehyde reactions.

The purpose of this study was to elucidate the chemical nature of the reactions between formaldehyde and proteins. Therefore, a set of model peptides was prepared and used to map systematically the different chemical modifications induced by formaldehyde treatment. The selected peptides can be divided into two groups (see Table 1): the first group had the amino acid sequence Ac-VELXVLL, in which one amino acid residue (X) varies and the remaining amino acid residues are non-reactive with formaldehyde. The second group was synthesised for studying the possible formation of intramolecular, formaldehyde-mediated cross-links between two reactive residues and contained peptides with the following sequence: Ac-LOENXLLZF-NH_2, where O, X and Z are either a (non-reactive) alanine residue or an arginine, lysine and histidine residue in different permutations (see Table 1). The reaction conditions were largely based on the detoxification process of diphtheria toxin for vaccine production (1). Especially, glycine was chosen to study in detail the cross-link reaction with peptides, because glycine is used as a reagent during the inactivation of diphtheria toxin by formaldehyde for the preparation diphtheria toxoid vaccines (6). The conversion of peptides was monitored by tandem reversed-phase liquid chromatography, electrospray ionisation mass spectrometry (LC-MS). In this paper we present an overview of the major conversion products resulting from reactions between model peptides and formaldehyde (in the absence and presence of glycine), several of which have not been identified before. Our data can be used for the prediction and identification of reactive sites in proteins after exposure to formaldehyde.

Materials and methods

Chemicals

Formaldehyde (37%), formic acid (99%), glycine, potassium dihydrogen phosphate ($KH_2PO_4 \cdot 3H_2O$) and dipotassium hydrogen phosphate ($K_2HPO_4 \cdot 3H_2O$) were purchased from Merck (Amsterdam, The Netherlands). Sodium cyanoborohydride ($NaCNBH_3$) was

Table 1. Peptides involved in this study and their mass increments after formaldehyde treatment under standard conditions[1)].

Peptide	Peptide sequence	Formaldehyde Δm (Da)	Formaldehyde/ $NaCNBH_3$ Δm (Da)	Formaldehyde/ glycine, Δm (Da)	Formaldehyde/ Ac-Arg-OMe Δm (Da)
1	Ac-VEL**A**VLL-OH	0	-[3)]	0	-
2	Ac-VEL**C**VLL-OH	30	-	30	242
3	Ac-VEL**D**VLL-OH	0	-	0	-
4	Ac-VEL**F**VLL-OH	0	-	0	-
5	Ac-VEL**H**VLL-OH	0	-	87	0
6	Ac-VEL**K**VLL-OH	0	28	0	254
7	Ac-VEL**M**VEL-OH[2)]	0	-	0	-
8	Ac-VEL**N**VLL-OH	0	-	87	0
9	Ac-VEL**P**VLL-OH	0	-	0	-
10	Ac-VEL**Q**VLL-OH	0	-	87	0
11	Ac-VEL**R**VLL-OH	30	-	99/198	0
12	Ac-VEL**S**VLL-OH	0	-	0	-
13	Ac-VEL**T**VLL-OH	0	-	0	-
14	Ac-VEL**W**VLL-OH	12/30	-	12/87	0
15	Ac-VEL**Y**VEL-OH[2)]	0	-	87/174	-
16	**L**AENALLAF-NH_2	12	28/26[4)]	12/99	-
17	Ac-LAENALLAF-NH_2	0	-	0	-
18	Ac-LAENALL**H**F-NH_2	30	-	87	-
19	Ac-LAEN**K**LLAF-NH_2	12/30	28	12/30	-
20	Ac-L**R**ENALLAF-NH_2	30	-	99/198	-
21	Ac-L**R**ENALL**H**F-NH_2	30	-	99/186/198/285	-
22	Ac-LAEN**K**LL**H**F-NH_2	12/30	28/26[4)]	12/30/87	-
23	Ac-L**R**EN**K**LLAF-NH_2	24	28/24[4)]	24/99/111/123/198/210	-
24	Ac-L**R**EN**K**LL**H**F-NH_2	24	28/24[4)]	24/99/111/123/153/186/ 198/21/285/297	-

[1)] See materials and methods for details.
[2)] The peptides Ac-VELMVLL-OH and Ac-VELYVLL-OH could not be obtained in acceptable purity. Therefore, the peptides Ac-VELMVEL-OH and Ac-VELYVEL-OH were synthesised.
[3)] Experiment was not performed.
[4)] Peptide products with these mass increases were formed after 48 h incubation with formaldehyde followed by incubation with $NaCNBH_3$.

obtained from Sigma (Zwijndrecht, The Netherlands). N^{α}-Acetylarginine methyl ester (Ac-Arg-OMe) was from Bachem Ag (Bubendorf, Switzerland). Dimethyl sulfoxide (DMSO) ultra grade was acquired from Acros Organics (‘s-Hertogenbosch, The Netherlands). Endoproteinase Glu-C was bought from Roche Applied Science (Almere, The Netherlands).

Peptides

Peptides (Table 1) were synthesised on 30 mmol scale by using an automated multiple peptide synthesiser, equipped with a 96-column reaction block (SYRO II, Fa.

MultiSynTech, Gmbh, Witten, Germany). Couplings were performed with fluorenyl methoxycarbonyl (Fmoc)-amino acid (90 mmol), benzotriazolyloxy-*tris*-[*N*-pyrrolidino]phosphonium hexafluorophosphate (90 mmol), and *N*-methylmorpholine (180 mmol). Single couplings were performed in cycles 1-9 and double couplings as from cycle 10. The Fmoc group was cleaved with piperidine/*N*,*N*-dimethylacetamide, 2/8 (v/v). Side-chain deprotection and cleavage from the solid support was effected with trifluoroacetic acid (TFA)/water, 95/5, v/v, except for cysteine-, methionine- and tryptophan-containing peptides, which were treated with TFA/ethanethiol, 95/5, v/v. The peptides were purified by reversed-phase (C8 column) high performance liquid chromatography and their identity was confirmed by LC-MS. Before use, peptides were dissolved in water or DMSO/water (50/50, v/v) to a final concentration of 10 mM.

Standard reactions with peptides

For the reaction of peptides with formaldehyde, 10 μl of a 10 mM peptide solution, 10 μl of 1 M potassium phosphate pH 7.2 and 5 μl of a second agent (1.0 M glycine, 1.0 M $NaCNBH_3$, 1.0 M Ac-Arg-OMe, or water) were added to 70 μl water. The reaction was started by adding 5 μl of an aqueous solution of 1.0 M formaldehyde. After mixing, the solution was incubated for 48 h at 35 °C. Samples were stored at –20 °C before analysis.

Variations in reaction conditions

The effect of different reaction conditions was investigated by varying the reaction time, pH, reagent concentrations and the moment of addition of $NaCNBH_3$. The reaction of peptides with formaldehyde and glycine was monitored for 6 weeks. Aliquots (10 μl) were taken after 2, 6, and 24 hours, 2, 6 and 24 days, and 6 weeks, and stored at –20 °C before analysis. To investigate the effect of pH, reactions were performed in potassium phosphate buffer with pH values of 5.2, 7.2, and 9.2. The influence of the concentration of the reagents on adduct formation was studied by varying the formaldehyde or the glycine concentration to final concentrations of 5, 50 and 500 mM. To determine internal cross-links in peptides, $NaCNBH_3$ was added 48 hours after formaldehyde addition.

Removal of excess formaldehyde

Removal of formaldehyde was performed on an HPLC-system equipped with a 10 cm long x 200 μm inner diameter column filled with Poros 10 R2 (5 μM; Perseptive Biosystems). The sample was diluted with water to a peptide concentration of 100 μM, and 10 μl of the diluted sample was trapped on the column. The column was rinsed for 10 min with solvent A (0.075% TFA in water) at a flow rate of 3 μl/min to remove formaldehyde. The peptide was eluted by a linear gradient from 0 % - 60 % solvent B (0.075% TFA in acetonitrile) in 25 min. The fraction containing the peptide was dried in a vacuum centrifuge (Concentrator 5301; Eppendorf), and dissolved in 100 μl water. Sample was stored at –20 °C before analysis.

Formaldehyde treatment of Ac-Arg-OMe

Formaldehyde, glycine and an arginine derivative, Ac-Arg-OMe, were dissolved or diluted in D_2O to final concentrations of 1.0 M. A reaction mixture was prepared by successively adding 400 µl glycine solution, 100 µl Ac-Arg-OMe solution and 200 µl formaldehyde solution to 300 µl D_2O. After each addition, the solution was homogenised by gentle mixing. The preparation was incubated for 48 h at 35 °C. Sample was stored at –20 °C before analysis.

Digestion by endoproteinase Glu-C

Peptides were digested by mixing 5 µl of 1mM peptide solution, 5 µl of 1.0 M potassium phosphate buffer pH 9.0, 1.0 µl of 1 µg/µl endoproteinase Glu-C solution and 39 µl of water, followed by incubation for 24 h at 37 °C. Subsequently, samples were stored at –20 °C before analysis.

Nano-electrospray MS

Analytes were diluted to a concentration of 10 µM in water containing 5% (v/v) DMSO and 5% (v/v) formic acid. Gold-coated nano-electrospray needle with an orifice of 1-2 µm inner diameter was loaded with 10 µl of the sample. A stable spray was obtained by an overpressure of 0.5 bar onto the needles, and adjusting the electrospray voltage to 0.75 kV. The capillary was heated to 150 °C. MS-spectra were acquired from m/z 50 – 2000, followed by successive stages of collision-induced dissociation (up to MS^4-measurements). The collision energies were optimised for each individual collision-induced dissociation mass analysis (between 30-35 %).

LC-MS

Peptide samples were analysed by nano-scale reversed phase liquid chromatography (HP 1100 Series LC system; Hewlett Packard GmbH, Waldbronn, Germany) coupled to electrospray mass spectrometry (LCQ™ Classic Quadrupole Ion Trap), essentially as previously described by Meiring et al. (23). Briefly, each peptide sample was diluted to a concentration of 0.1 µM in water containing 5% (v/v) DMSO and 5% (v/v) formic acid. An injection volume of 10 µl was used for analysis. To desalt the samples for MS analysis, analytes were trapped on a 15 mm long x 100 µm inner diameter trapping column with Aqua C18 (5 µm; Phenomenex) at a flow rate of 3 µl/min and by using 100% solvent A (0.1 M acetic acid in water) as eluent for 10 min. Then, analytes were separated by reversed-phase chromatography by using a 25 cm long x 50 µm inner diameter analytical column with Pepmap (5 µm; Dionex) at a flow rate between 100-125 nl/min. A linear gradient was started from 10 % solvent B (0.1 M acetic acid in acetonitrile) to 60 % solvent B in 25 min. Next, the columns were equilibrated in 100% solvent A for 10 min.

The analytes were measured in the MS^1-mode (m/z 400 – 2000) to determine the mass

increase and conversion of peptides after incubation with formaldehyde. The heated capillary was set to 150 °C and electrospray voltage to 1.6-1.7 kV. A second LC-MS measurement was performed to obtain detailed sequence information. Therefore, the peptides were analysed by data dependent scanning comprising a MS^1-scan (m/z 400 – 2000) followed by collision-induced dissociation of the most abundant ion in the MS^1-spectra. The collision energy was set on 35%.

Results and Discussion

Establishment of reaction conditions
A set of synthetic peptides (Table 1) was used to investigate the reactivity of amino acid residues reacting with formaldehyde. The reaction was monitored during 6 weeks by LC-MS. In general, shorter exposure of the peptides sensitive to formaldehyde resulted in lower conversions. After a reaction time of 48 hours, all modifications that were observed in this introductory study were detectable by using LC-MS-analysis. Variation in the pH showed that reactions did not occur below pH 5, and that a maximal conversion rate was reached above pH 7. Furthermore, the conversion of the peptides was proportional with the reactant concentration. Based on these experiments, we used the following standard reaction conditions in rest of this study (unless stated otherwise): 50 times excess of formaldehyde (and glycine) with regard to the peptide concentration, incubation at pH 7.2 and 35 °C for 48 hours.

Formation of methylol and imine adducts
Peptide 1 was designed with amino acids residues, which were expected not to react with formaldehyde (Table 1). Indeed, LC-MS-analyses showed that peptide 1 was not modified after incubation with formaldehyde. On the other hand, peptides containing a cysteine (peptide 2), arginine (peptides 11 and 20), tryptophan (peptide 14), histidine (peptide 18) or lysine residue (peptide 19) gave products with a mass increase of a 30 Da. A second modification was observed in peptides, containing a tryptophan or a lysine residue (peptides 14 and 19, respectively). This modification caused a mass increase of 12 Da. Unexpectedly, and in contrast with the results of histidine-containing peptide 18 and lysine-containing peptide 19 (showing mass increases of 12 and/or 30 Da), formaldehyde treatment of peptide 5 (containing histidine) and peptide 6 (containing lysine) did not yield detectable amounts of reaction products. Nonetheless, a formaldehyde-glycine adduct could be attached to the histidinyl in peptide 5, and the lysyl in peptide 6 could react with formaldehyde and $NaCNBH_3$. Thus, both residues were reactive with formaldehyde (see Table 1). Therefore, we assume that the reaction equilibrium towards the methylol and imine adduct depends on the amino acid sequence.
The increase of 30 Da is an indication for the formation of a methylol group (Scheme 1,

reaction 1). Under standard reaction conditions, the conversion varied between 3-22%, depending on the peptide. The formation of a methylol adduct to peptides is a reversible reaction, because the conversion of the arginyl peptide 20 was reduced form 17% to 4.5% after removal of free formaldehyde.

Structural analysis of reaction products

MS^2-analyses were performed on formaldehyde-treated peptides to confirm that the methylol was located on cysteine, histidine, lysine and arginine residues (peptides 2, 18, 19, and 20). The spectra revealed that peptide fragments were present with a mass increase of 30 Da, if they still contained a cysteine, arginine, or a histidine residue, whereas peptide fragments lacking these residues had the same mass as the corresponding MS^2-fragments of non-treated peptides. MS^2-measurements on the peptide containing a methylol on the lysine residue showed only peptide fragments with a mass increase of 12 Da instead of 30 Da, apparently due to dehydration of the methylol group (Scheme 1, reaction 2). The methylol located on a tryptophan residue could not be verified by MS^2-measurements, possibly because of the low conversion (3%). In conclusion, side chains of cysteine, histidine, lysine, arginine, and tryptophan residues can form methylol groups in the presence of formaldehyde.

Two possible reaction products could account for a mass increase of 12 Da found in formaldehyde-treated peptides 14 and 19: the formation of an imine or a methylene bridge (Scheme 1, reaction 2 and 3). These possibilities were studied by MS^2-measurements by generating immonium ions as a consequence of peptide fragmentation (24). MS^2-measurements performed on formaldehyde-treated peptide 14 showed that the mass increase of 12 Da was located on the tryptophan residue. The typical immonium ion of tryptophan (159 Da) was lost after the reaction with formaldehyde and a new fragment appeared (171 Da), indicating that the tryptophan residue was modified. No other new masses were detected, excluding the possibility that cross-links were formed between two residues. The proposed structure of the modified tryptophan residue is given in figure 1.

Pep
N^+
CH_2

Figure 1. The imine adduct of a tryptophan residue formed after formaldehyde treatment.

MS^2-measurements were also performed on formaldehyde-treated peptide 19 to determine the type of modification formed. The spectra revealed that during formaldehyde incubation a fragment with a mass of 84 Da disappeared and a fragment of 113 Da appeared. The fragment of 84 Da can be attributed to an immonium ion of an unmodified lysine residue. Another expected immonium ion of 101 Da was not found, but in general this fragment is

less frequently observed than the immonium ion of 84 Da, which lacks the ε-NH_2 group. The characteristic immonium ion of 113 Da, which was found after formaldehyde treatment, is indicative of the formation of a Schiff-base (Figure 2). A second confirmation for the presence of a Schiff-base in peptide 19 was the reaction with $NaCNBH_3$, which was added 48 hours after the incubation with formaldehyde. The ε-amino group of lysine was quantitatively converted to a dimethylated amine with a mass increase of 28 Da (Scheme 2). In conclusion, side chains of tryptophan and lysine residues can form imines during incubation with formaldehyde.

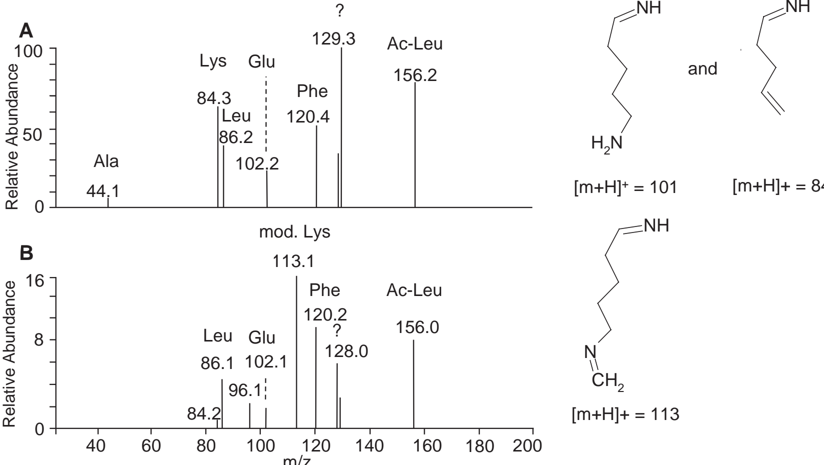

Figure 2. MS^2-spectra of peptide 19 containing a lysine residue. Immonium ions of the lysine residue have typical masses of 101 and 84 Da. The mass of 101 Da is in general less frequently observed. Spectrum B shows the immonium ions of the modified peptide. An immonium ion with the particular mass of 113 corresponds to a modified lysine residue. Corresponding structures of the lysine immonium ions are shown on the right.

$$3\ R{-}NH_2 + 6\ HCHO + 2\ NaCNBH_3 \longrightarrow 3\ R{-}N(CH_3)_2 + 2\ HCN + 2\ NaH_2BO_3$$

$$(\Delta m = +28)$$

Scheme 2. Reduction of primary amino groups by adding formaldehyde and $NaCNBH_3$ (7, 8).

Intramolecular cross-links

Peptide 16 containing a primary N-terminal amino group was almost completely converted within 48 hours into an adduct with a mass increment of 12 Da. According to the literature, formaldehyde (and acetaldehyde) can form a stable methylene bridge in such peptides, as

determined by NMR and MS-measurements (25-27). The resulting ring structure is a 4-imidazolidinone (Scheme 3, reaction 1). To confirm the formation of a 4-imidazolidinone, we added $NaCNBH_3$ to the peptide after 48 hours incubation with formaldehyde. This resulted in the formation of a peptide adduct with a mass increase of 26 Da (Scheme 3, reaction 2), indicating that an *N*-methyl-4-imidazolidinone had indeed formed. Normally, when adding formaldehyde and $NaCNBH_3$ simultaneously, N-terminal amino groups are reduced into a dimethylated amine. Indeed, a mass increase of 28 Da was then shown for peptide 16.

Scheme 3. Modifications of peptide 22 containing a free N-terminus. A 4-imidazolidinone adduct was formed after adding formaldehyde to a peptide, probably via a Schiff-base intermediate [1]. The imidazolidinone could be reduced by adding $NaCNBH_3$ after 48 hours incubation of peptide 22 with formaldehyde. An *N*-methyl-4-imidazolidinone was formed [2]. A glycine-formaldehyde adduct could be attached to the imidazolidinone [3].

The formation of intramolecular cross-links was also expected for peptides 21 to 24, because they contain two (peptide 21 to 23) or three amino acid residues (peptide 24) that are reactive with formaldehyde, i.e. they contain lysine, arginine and/or histidine residues. Under standard reaction conditions, peptide 21 showed one adduct with a mass increase of 30 Da after formaldehyde treatment. This suggests that one methylol adduct was formed, probably on the arginine or the histidine residue. A product with a mass increase of 60 Da was also expected, but could not be detected. When increasing the formaldehyde

concentration to 500 mM, a reaction product was observed with a mass increase of 60 Da, indicating that two methylol groups were attached to the peptide. However, no intramolecular cross-link was formed in this peptide, because in that case a mass increase of 12 Da was expected.

Formaldehyde-treated peptide 22 showed, beside the product with a mass increase of 30 Da, two minor products with a mass increase of 12 Da (Figure 3). These minor products might be due to the formation of a Schiff-base located on the lysine residue or a methylene bridge between lysine and histidine residues. Addition of $NaCNBH_3$ to the formaldehyde-treated peptide yielded two products: small amounts of a peptide adduct with a mass increase of 26 Da and a larger amount with a increment of 28 Da. The mass increase of 28 Da can be explained by the formation of dimethylated lysine, whereas the increase of 26 Da presumably reflects a product with an intramolecular cross-link between the lysine and the histidine residue.

Both formaldehyde-treated peptides 23 and 24 showed two LC-peaks of adducts with a

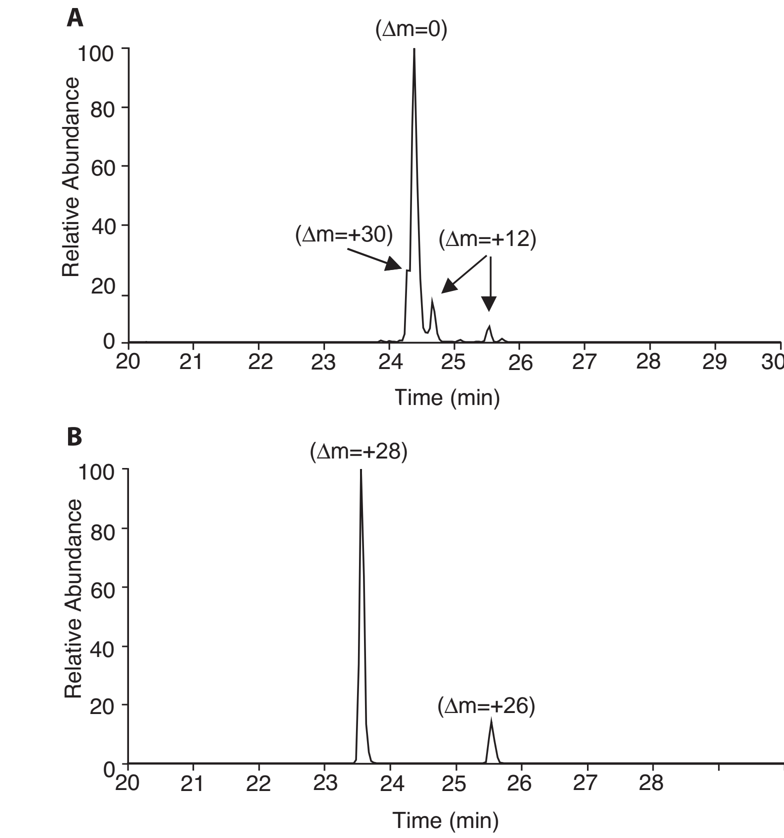

Figure 3. LC-MS chromatograms of formaldehyde-induced modifications in peptide 22. (A) after 48 h incubation with formaldehyde. (B) after 48 h incubation with formaldehyde followed by 48 h incubation with $NaCNBH_3$.

mass increase of 24 Da. These products could not be reduced by $NaCNBH_3$, which suggests that two methylene bridges had been formed between the side chains of lysine and arginine. The proposed structures are given in figure 4. The following experiment was performed to verify this hypothesis. Peptide 24 contains a glutamic acid residue between the arginine and the lysine residue (Table 1), which allows cleavage of the peptides by endoproteinase Glu-C. So, the original and the formaldehyde-treated peptide 24 were both incubated with proteinase Glu-C. The original peptide was completely hydrolysed by proteinase Glu-C, yielding two fragments with expected m/z of 770 and 459 Da. On the other hand, the formaldehyde-treated peptide 24 with a mass of 1234 Da was partially converted to a product with a mass of 1252 Da. The mass increase of 18 Da indicates that the peptide bond was hydrolysed at the carboxylic site of the glutamic acid residue and that the two parts were still coupled to each other via cross-links between the lysine and arginine residues. This strongly supports the proposed structures given in figure 4.

In contrast to previous studies with amino acids (18, 19), intermolecularly cross-linked peptides were not detected. This may be due to differences in reaction conditions and

Figure 4. Modification of peptide 23 and 24. Two different structures were presumably formed after incubation of these peptides with formaldehyde. Two methylene bridges were formed between the lysine residue and the arginine residue. The peptide bond at the C-terminal site of a glutamine residue can be cleaved with proteinase Glu-C.

possible lower reactivity of peptides as compared to free amino acids. Unfortunately, the poor solubility of the peptides did not allow us to investigate whether cross-linking between two peptides occurs at higher concentrations. Increasing reaction time or mixing peptides with different reactive residues did not result in any intermolecular cross-linking.

Cross-links between glycine and peptides

From the previous experiments, it was shown that cross-links were formed between lysine and histidine residues or between lysine and arginine residues after formaldehyde treatment. To investigate whether formaldehyde can form methylene bridges between other amino acid residues, all peptides were individually incubated for 48 hours with a fifty-fold excess of formaldehyde and glycine. MS^1-analysis showed that glycine and formaldehyde reacted to form a reactive imine adduct with an ion mass of 88 Da. This glycine/formaldehyde adduct reacted with peptides containing a histidine (peptide 5 and 18), asparagine (peptide 8), glutamine (peptide 10), or tryptophan residue (peptide 14), yielding products with a mass

increase of 87 Da. This can be explained by the coupling of glycine to the peptides via a methylene bridge (Table 2).

The peptide with a tyrosine residue (peptide 15) gave two products with an increase of 87 Da and 174 Da, which means that one or two glycine/formaldehyde adducts were coupled to the peptide, most probably to the ortho positions of the phenolic group (Table 2). This type of reactions is known as the Mannich synthesis (21, 28).

Table 2. Proposed structures of glycine-formaldehyde adducts attached to different amino acid residues present in peptides.

Residue	Mass increase (amu)	Modification
Asparagine and glutamine	87	
Histidine	87	
Tryptophan	87	and/or
Tyrosine	87 and/or 174	and/or
Arginine	99	and/or
	198	
N-terminal amino group	99	

Glycine was also coupled by formaldehyde to the N-terminal amino group of peptide 16, which gave the peptide a mass increase of 99 Da. The formation of this adduct occurs presumably via two steps: first, a 4-imidazolidinone is formed and then the glycine is attached via a methylene bridge to this 4-imidazolidinone. The proposed structure is given in scheme 3, reaction 3.

Arginine-containing peptides were also modified by formaldehyde and glycine. Peptide products were found with mass increases of 99 and 198 Da. The mass increase can be explained by the coupling of one or two glycine molecules to the peptide via two methylene bridges (see Table 2). To verify that this modification occurs specifically on the arginine residue, an arginine derivative, Ac-Arg-OMe, was treated with formaldehyde and glycine. The two main products from Ac-Arg-OMe had the same mass increase of 99 and 198 Da. Their proposed structures are given in scheme 4. MS^4-measurements were performed on both products with ion masses of 330 Da and of 429 Da. The product with ion mass of 330 Da was degraded after four repeated fragmentations into fragment ions with m/z of 243, 214, and 172 Da. The product with ion mass of 429 Da was fragmented to ions with m/z of 342, 255, 226, and 184 Da. The possible structures of the fragment ions are given in scheme 4.

Unexpectedly, formaldehyde did not cross-link detectable amounts of glycine to peptides 2, 6 and 19 containing a cysteine or a lysine residue, whereas these types of cross-links have been described in several articles (1, 8, 22, 29, 30). Prolonging the reaction time or increasing the formaldehyde and glycine concentration did not have any effect. The peptide samples were normally analysed by LC-MS in an acidic environment. Since the methylene bridge between two amino groups might be unstable in an acidic environment (31), the samples were also measured at neutral pH by static nano-electrospray ionisation analysis. No modifications were found in these peptides. These outcomes are in contrast to the results of others who treated 1,3,-diaminopropane and cysteine with formaldehyde. Methylene bridges were formed between the two amino groups in 1,3,-diaminopropane, and between amino and thiol groups of cysteine (22, 30). However, both 1,3,-diaminopropane and cysteine formed intramolecular cross-links, whereas in our experiments intermolecular cross-links have to be formed between the peptide and glycine. Follow-up studies with a peptide containing two lysine residues may shed light upon these different observations.

MS-analyses of peptides 21-24 treated with formaldehyde and glycine demonstrated that several products were formed with different masses (Table 1). These peptides contain two or three residues that can react with formaldehyde. The determined masses can be explained as a combination of methylol adducts, imines, and intramolecular and intermolecular cross-links.

The conversion of peptides after glycine/formaldehyde treatment might predict the reaction rate of formaldehyde-induced intramolecular cross-links. Especially, intramolecular cross-links in proteins are initiated by reaction of formaldehyde with lysine residues. Subsequently, the adducts probably form cross-links with reactive residues in their direct environment, and especially with the residues that have the highest reactivity. Therefore, the

MS[1] → $- C_3H_5NO_2$ → MS[2] → $- CH_3N$ →

m/z = 330.1 m/z = 234.0

MS[3] → $- CH_2N_2$ → MS[4]

m/z = 214.0 m/z = 170.2

(A)

MS[1] → $- C_3H_5NO_2$ → MS[2] → $- C_3H_5NO_2$ →

m/z 429.0 m/z 342.0

MS[3] → $- CH_2N_2$ → MS[4]

m/z 255.0 m/z 226.1

(B)

Scheme 4. MS analysis of two products formed during incubation of N^{α}-Acetylarginine methyl ester (Ac-Arg-OMe) with formaldehyde and glycine. The products (MS[1] in A and B) were fragmented by the mass spectrometer in four successive steps. The m/z of the products and the fragments were measured. Their possible structures are given in the table.

conversion of peptides was determined after 48 h incubation with formaldehyde and glycine (Table 3). The results show that the formaldehyde-glycine adduct was rapidly attached to free N-terminal amino groups, to arginine and tyrosine residues of peptides, and to a lesser extent to asparagine, glutamine, histidine, and tryptophan residues.

Table 3. Conversion of peptides to a glycine-formaldehyde adduct after 48 h incubation with formaldehyde[1].

Residue	Δm (Da)	Conversion (%)
Asparagine	87	4.0 ± 1.8
Arginine	99	56 ± 13
	198	41 ± 14
Glutamine	87	3.6 ± 1.6
Histidine	87	6.6 ± 1.9
Tryptophan	87	4.7 ± 1.5
Tyrosine	87	62 ± 4
	174	5.2 ± 1.3
N-terminal amino group	12	76[2]
	99	13[2]

[1] (mean ± SD; n=3).
[2] (n=1).

Cross-links between Ac-Arg-OMe and peptides

In theory, the cross-link reactions between glycine and peptides caused by formaldehyde can only occur via an imine. To verify this, peptides 5, 6, 11, and 14, containing a histidine, lysine, arginine and tryptophan residue, respectively, were incubated with formaldehyde and Ac-Arg-OMe for 48 h. Ac-Arg-OMe was only cross-linked to peptide 6 and gave the peptide a mass increase of 254 Da. This observation indicates that two methylene bridges were formed between the primary amine group of the lysine residue in peptide 6 and the arginine derivative (Figure 5A). Peptides containing a histidine, arginine or a tryptophan residue did not react with formaldehyde and Ac-Arg-OMe, confirming that that the reaction occurred via an Schiff-base.

Additionally, peptide 2 with a cysteine residue was treated with formaldehyde and Ac-Arg-OMe to demonstrate that thiol groups can also form cross-links with arginine residues. A product with mass increase of 242 Da was found, indicating that one methylene bridge had been formed between the thiol group of peptide 2 and the arginine derivative (Figure 5B). It has been reported in the literature that formaldehyde can react with the α-amino group and β-thiol group of the N-terminal cysteines under formation of thiazolidine derivatives (32, 33). From these reports and our present data we conclude that cysteine residues can form cross-links at least with arginines and with N-terminal amino groups as a result of formaldehyde treatment.

Figure 5. (A) Modifications of peptide 6 after incubation with formaldehyde and N^{α}-Acetylarginine methyl ester (Ac-Arg-OMe). (B) Modifications of peptide 2 after incubation with formaldehyde and Ac-Arg-OMe.

Conclusions

This study has demonstrated that, depending on their sequence, peptides undergo a great diversity of chemical modifications after formaldehyde treatment. The modifications can be divided in three types: (i) methylol groups, (ii) Schiff-bases and (iii) methylene bridges. The formation of methylol and Schiff-bases is reversible, and therefore these compounds are in general hard to detect. Still, methylol and Schiff-base derivatives could be demonstrated in several peptides by using LC-MS. They were located on residues with an amino or a thiol group.

The most important modification of peptides (and proteins) induced by formaldehyde is the formation of stable methylene bridges. In this study we showed that only primary amino and thiol groups primarily react with formaldehyde, and form cross-links in a second step with several other amino acid residues, i.e. with arginine, asparagine, glutamine, histidine, tryptophan, and tyrosine residues. In contrast to these cross-link reactions, no methylene bridges were formed between two primary amino groups. Moreover, Ac-Arg-OMe was not coupled to asparagine, glutamine, histidine, or tryptophan residues of a peptide, indicating that only primary amino groups can form intermolecular cross-links with these reactive residues.

To discriminate between the formation of a Schiff-base or a methylene bridge, $NaCNBH_3$ was used. The reaction with formaldehyde and $NaCNBH_3$ is specific for primary amino groups; for peptides or proteins only the N-terminus and lysine residues are converted to dimethyl amino groups (7). In peptides, this conversion result a mass increase of 28 Da.

Cross-links between a lysine and a histidine residue were demonstrated by adding $NaCNBH_3$, resulting in a mass increase of 26 Da.
In conclusion, with the present study we have provided a detailed overview of possible chemical modifications of each individual amino acid residue caused by formaldehyde. Furthermore, the relative reactivity of the residues to form a particular cross-link was elucidated. Although identification of all intramolecular cross-links of formaldehyde-treated proteins still will be a tremendous job, the data from this study can be helpful to interpret peptide maps. In addition, if the local environment of the reactive residues is known (e.g., through X-ray crystallography or NMR studies), our data may be useful to predict the modifications in formaldehyde-treated proteins.

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Chapter 6

Identification of formaldehyde-induced modifications in proteins: reactions with insulin

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Abstract

Proteins undergo a large variety of chemical modifications in the presence of formaldehyde, such as the formation of methylol groups, Schiff-bases and methylene bridges. In our previous paper (B. Metz et al, J Biol Chem (2004) 279, 6235-43), we showed which formaldehyde-induced modifications of amino acid residues occur by using synthetic model peptides. The purpose of the present study was to use the acquired knowledge for mapping the formaldehyde-induced modifications in a small model protein, insulin. In theory, insulin has sixteen potential sites that can be modified by formaldehyde and glycine. To study which modifications actually occur, insulin was treated with excess formaldehyde (CH_2O) or deuterium-labeled formaldehyde (CD_2O) in combination with glycine. The mixture of CH_2O-treated and CD_2O-treated insulin was digested by proteinase Glu-C and fragments were analyzed by liquid chromatography-mass spectrometry (LC-MS). The use of deuterium-labeled formaldehyde simplified the recognition of formaldehyde adducts. For ten out of the sixteen potentially reactive sites, the location and type of modifications could be assigned. To confirm the assigned structures, MS-measurements with collision-induced dissociation (MS/MS) were performed on insulin fragments with a sufficient intensity in MS. The results of the MS/MS-analyses agreed excellently with the assignments. No modifications were observed for six other residues. In accordance with our previous study with model peptides, arginine, tyrosine and lysine residues were very reactive, but formaldehyde and glycine either did not or slightly converted the asparagine, glutamine, and histidine residues. The expected imidazolidinone adducts on the N-termini of insulin were not detected, probably as a result of steric hindrance. This study indicates that the protein conformation affects the accessibility and reactivity of formaldehyde susceptible residues in insulin. The approach that has been followed in this study may be generally applicable to formaldehyde treated proteins like several vaccine antigens.

Introduction

Formaldehyde is a well-known cross-linking agent that is often applied in the biochemical and pharmaceutical field to inactivate, stabilize, or immobilize proteins. Formaldehyde is utilized for the preparation of vaccines (1-4), for isotope-labeling of proteins (5-7), and for studying protein-protein interactions (8-10). Numerous chemical modifications occur in proteins during the treatment with formaldehyde. Extensive studies performed with amino acids or derivatives thereof revealed that formaldehyde can modify several amino acid residues in proteins (11-15). The reaction of formaldehyde with a peptide or protein starts with the formation of unstable methylol adducts on amino groups. The methylol groups partially dehydrate, yielding labile Schiff-bases, which can form cross-links with several amino acid residues. Formaldehyde-induced modifications in proteins are hardly investigated. Only a few articles describe investigations of formaldehyde-induced modifications in proteins (16-19), but the nature of each specific modification and the exact location of the modified residues were not identified.
In a previous study with model peptides, we have elucidated formaldehyde-induced modifications and the reactivity of each amino acid residue (20). This study has revealed that methylol adducts were formed with arginine, cysteine, histidine, lysine and tryptophan residues, whereas Schiff-bases were found with lysine and tryptophan residues (table 1). Moreover, stable cross-links were detected between primary amine groups and several amino acid residues, including arginine, asparagine, glutamine, histidine, tryptophan, and tyrosine. The N-terminal residue are modified after formaldehyde treatment resulting in an imidazolidinone (20-23). However, the effect of the protein conformation on the reactivity of amino acids residues can hardly be studied in model peptides. In addition, the position and local environment of each reactive amino acid in the protein may affect the reactivity.
The aim of the present study was to find formaldehyde-induced modifications in proteins by applying the knowledge obtained from our previous study with model peptides. For this

Table 1. Mass increase due to formaldehyde modifications of reactive amino acid residues[a].

Residue	methylol ΔM	imine ΔM	Formaldehyde-glycine adduct ΔM
Arginine	30	-	99/198
Asparagine	-[b]	-	87
Cysteine	30	-	-
Glutamine	-	-	87
Histidine	30	-	87
Lysine	30	12	-
Tryptophan	30	12	87
Tyrosine	-	-	87/174
N-terminal amino acid	-	12	99

[a] Results from studies with peptides (20).
[b] - = No modification observed.

purpose, insulin from bovine pancreas was used as a model protein. Insulin composes of two polypeptide chains: (i) the A-chain consists of 21 amino acid residues and (ii) the B-chain contains 30 amino acid residues; the two chains are interconnected via two disulfide linkages between residues $C^{A7} – C^{B7}$ and between $C^{A20} – C^{B19}$ (Figure 1). In this study, insulin was treated with either native formaldehyde or deuterium-labeled formaldehyde (CH_2O vs. CD_2O) and glycine. The use of these ”light” and “heavy” isotopes facilitates the unequivocal identification of chemical modifications. After formaldehyde treatment, a mixture of equimolar amount of CH_2O-treated and CD_2O-treated insulin was enzymatically digested into small peptides, which were subsequently analyzed by reversed-phase liquid chromatography, electrospray ionization mass spectrometry (L C-MS). Using this approach, we were able to detect the nature and location of formaldehyde-induced modifications in insulin.

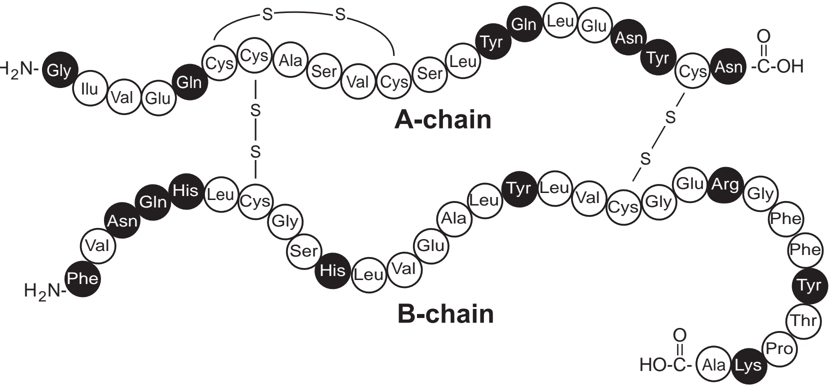


Figure 1. The primary structure of bovine insulin. The A-fragment and B-fragment of insulin are connected to each other via two disulfide bridges. Furthermore, the A-fragment has an intrachain disulfide bridge. In theory residues given in black are reactive to formaldehyde.

Materials and methods

Chemicals

Formaldehyde (37% in water with 10% methanol), formic acid (99%), glycine, potassium dihydrogen phosphate ($KH_2PO_4{\cdot}3H_2O$) and dipotassium hydrogen phosphate ($K_2HPO_4{\cdot}3H_2O$) were purchased from Merck (Amsterdam, The Netherlands). Formaldehyde-D_2 (CD_2O) was delivered from C/D/N Isotopes Inc. (Utrecht, The Netherlands). Insulin from bovine pancreas, DL-dithiotreitol (DTT) and sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma (Zwijndrecht, The Netherlands). Endoproteinase Glu-C was bought from Roche Applied Science (Almere, The Netherlands).

Table 2. Composition of reaction mixtures used in this study.

Mixture		Reaction step 1			Reaction step 2	
	Insulin (0.35mM) µl	formaldehyde (1M) µl	glycine (1M) µl	$NaCNBH_3$ (1M) µl	formaldehyde (1M) µl	glycine (1M) µl
1	3.36	320	320	-	-	-
2	3.36	320	-	320	-	-
3	3.36	320	-	320	320	320

- = not applicable.

Reactions of insulin with formaldehyde

Three different reactions with insulin were performed in this study: (i) insulin with formaldehyde and glycine, (ii) insulin with formaldehyde and $NaCNBH_3$, and (iii) insulin with formaldehyde and $NaCNBH_3$ followed by the reaction with formaldehyde and glycine. For each reaction, two identical mixtures were prepared except for the type of formaldehyde: either native formaldehyde (CH_2O) or deuterium-labeled formaldehyde (CD_2O) was used. Prior to the reactions, insulin was dissolved in 10 mM potassium phosphate pH 8.5 to a final concentration of 0.35 mM, formaldehyde (CH_2O or CD_2O), glycine and $NaCNBH_3$ to a concentration of 1.0 M. The compositions of the reaction mixtures are given in table 2. The final concentration of insulin during the first reaction step was 0.30 mM and the final concentrations of formaldehyde, glycine and $NaCNBH_3$ were 80 mM. After mixing, the solution was incubated for 1 week at 35 °C. Samples were extensively dialyzed against 10 mM potassium phosphate pH 8.5 (MWCO 1000). For reaction 3, formaldehyde (CH_2O or CD_2O) and glycine were added to the dialyzed mixture resulting from the incubation with formaldehyde (CH_2O or CD_2O, respectively) and $NaCNBH_3$ (Table 2). The solution was incubated for 1 week at 35 °C, and the samples were dialyzed again. The product in mixture 3 is a result of reaction 1 and 2. After incubation and dialysis, equalmolar amounts of CH_2O-treated and CD_2O-treated insulin were mixed. All samples were stored at 4 °C prior to digestion with proteinase Glu-C.

Digestion by endoproteinase Glu-C

Non-treated insulin and formaldehyde-modified insulin samples were digested by mixing 50 µl of these samples with 5 µl of 1.0 M potassium phosphate buffer pH 9.0, 1.0 µl of 1 µg/µl endoproteinase Glu-C solution and 44 µl of water. The mixtures were incubated for 24 h at 37 °C. To reduce disulfide bonds, 1 µl of 0.1 M DTT was added to the digests and the samples were incubated for 1 h at 37 °C. Subsequently, the samples were stored at –20 °C prior to LC-MS analysis.

Nano-electrospray MS

Insulin was diluted to a concentration of 1 µM in water containing 5% (v/v) DMSO and 5%

(v/v) formic acid. Gold-coated nano-electrospray needles (length 600-700 μm; inner diameter 1-2 μm; home made) were loaded with 10 μl of the sample. The protein solution was analyzed by electrospray ionization mass spectrometry using a Q-TOF Ultima API mass spectrometer (Waters, England). MS-scans were obtained form m/z 350 – 2000 amu. Spectra were deconvoluted using the MaxEnt 1 tool in the MassLynnx MS software (Waters).

LC-MS

Protein digests were analyzed by nano-scale reversed phase liquid chromatography electrospray ionization mass spectrometry (Q-TOF Ultima API), essentially as previously described by Meiring et al. (24). Briefly, each digested sample was diluted in water containing 5% (v/v) DMSO and 5% (v/v) formic acid to a concentration, corresponding to 2.5 nM of the original insulin concentration. An injection volume of 10 μl was used for analysis. Analytes were trapped on a 15 mm long x 100 μm inner diameter trapping column with Aqua C18 (5 μm; Phenomenex) at a flow rate of 3 μl/min with 100% solvent A (0.1 M acetic acid in water) as eluent for 10 min. Then, analytes were separated by reversed-phase chromatography by using a 25 cm long x 50 μm inner diameter analytical column with Pepmap (5 μm; Dionex) at a flow rate of 125 nl/min. A linear gradient was started from 5 % solvent B (0.1 M acetic acid in acetonitrile) to 60 % solvent B in 55 min.

The digested peptides were measured in the MS-mode (m/z 350 – 2000) to determine the masses of peptides in the mixture. The mass spectrometer was adjusted to following conditions: the electrospray voltage was set to 2.3 kV, the TOF voltage to 9.1 kV, and the MCP voltage to 1.8 kV. Peptides that contain formaldehyde modifications typically appeared as mass spectral doublets as a result of the use of ”light” (CH_2O) and ”heavy” (CD_2O) formaldehyde. Based on the MS-results, a list of masses was compiled of formaldehyde-modified peptides, which were measured in a second run by LC-MS/MS to obtain sequence information. Therefore, the peptides were analyzed by data dependent scanning comprising a survey MS-scan (m/z 350 – 2000) followed by collisional activated decomposition (CAD) of the abundant ion in the MS-spectra from the compiled list. The collision energy was set between 15 and 25 V.

Results and Discussion

Characterization of untreated insulin

Bovine insulin was chosen as a small model protein to study the possible reactions of formaldehyde with amino acid side chains of proteins. The purity of the insulin was determined by nano-electrospray MS analysis. The major compound had a mass of 5729 Da, close to the theoretical mass of bovine insulin (5730 Da). Furthermore, three other masses of 5659, 5792 and 5855 Da were observed with intensities of 9%, 33% and 7%,

respectively, compared to mass of 5729 Da. The mass of 5659 Da can be ascribed to insulin lacking the alanine residue at the C-terminus of the insulin. The other two masses can be explained by the binding of one or two Zn^{2+} ions ($\Delta M = 65 - 2$ Da). In the presence of zinc, insulin associates into hexameric complexes (25). Based on mass spectra, the purity of the insulin was estimated higher than 95%.

Proteinase Glu-C, which cleaves at the C-terminal sites of glutamate and aspartate (26, 27), was used to digest insulin into small peptides. In theory, complete cleavage of the insulin and reduction of disulfide bridges will result in six non-overlapping peptides (Table 3). However, partial digestion and incomplete reduction of S-S bridges results in formation of extra peptides. Eight masses were measured by LC-MS analysis that could be ascribed to insulin-derived peptides (Table 3). On the other hand, masses according to peptide 4 and 6 were missing, but masses of overlapping peptides were observed. These peptides of insulin were partially cleaved or contained a disulfide bridge. Moreover, four masses were observed that were 16 Da heavier than the theoretical mass. This mass increase indicates oxidation of a susceptible residue, such as cysteine, histidine or tyrosine residues (28). Also a few masses with minor intensities were found that could not directly be related to insulin (not shown). Probably, these peptides either originated from (i) miss cleavages of proteinase Glu-C, or (ii) autocleavage of proteinase Glu-C.

Altogether, the digestion of insulin with proteinase Glu-C resulted in a total number of eight peptides that cover the whole protein. In principle, this permits mapping of formaldehyde-induced modifications on the entire insulin molecule.

Table 3. List of expected and detected insulin-derived peptides after digestion of insulin with proteinase Glu-C.

Peptide	Fragment	Sequence	MH^+ (Da)[a)]
A-fragment			
1	A^1 - A^4	GIVE	417.2 (0.0)
2	A^5 - A^{17}	QCCASVCSLYQLE	-[b)]
3	A^{18} - A^{21}	NYCN	-
B-fragment			
4	B^1 - B^{13}	FVNQHLCGSHLVE	1482.7 (0.0)
5	B^{14} - B^{21}	ALYLVCGE	867.4 (0.0)
6	B^{22} - B^{30}	RGFFYTPKA	1086.6 (0.0)
Peptides with disulfide-links			
7	A^5 - A^{17}	QCCASVCSLYQLE[c)]	1444.6 (0.0)
8	A^5 - A^{17} and B^1 - B^{13}	QCCASVCSLYQLE - FVNQHLCGSHLVE	2924.6 (0.3)
9	A^{18} - A^{21} and B^{14} - B^{21}	NYCN - ALYLVCGE	1377.6 (0.0)

[a)] Seven masses were observed that could be ascribed to peptides from bovine insulin. Deviation from the calculated masses given between brackets.

[b)] - = fragment not observed.

[c)] Disulfide links are formed between cysteine residues resulting in a mass decrease of 2 Da.

Formaldehyde/glycine-induced modifications in insulin

In our previous paper (20), we showed that nine different amino acid residues in proteins are modifiable by formaldehyde and glycine (Table 1). According to that study, sixteen amino acid residues in insulin can be modified, if they are easily accessible to formaldehyde and glycine (Figure 1). However, the protein conformation might influence the reactivity of each reactive residue. LC-MS analyses of mixtures of CH_2O/glycine-treated and CD_2O/glycine-treated insulin showed twenty-two peptide pairs; each pair indicating the presence of one or more formaldehyde-induced modifications (Table 4). The peptide pairs had a mass difference of n*2 Da, with n corresponding to the number of incorporated formaldehyde residues (see Figure 2 for representative examples). The number of formaldehyde molecules incorporated per peptide ranged from one to six. Sixteen masses could be ascribed to modified peptide fragments from insulin. The assignment was based on the insulin sequence and the earlier described formaldehyde modifications (20). Moreover, the number of incorporated formaldehyde molecules corresponded with the prediction. Seven observed masses can be attributed to products resulting from incomplete cleavage of insulin by proteinase Glu-C. Six masses of formaldehyde-modified peptides could not be explained, so far.

The assigned peptide sequences could only be verified by MS/MS-analyses, if their intensities were high (relative abundance >10^3). MS/MS-measurements revealed the exact location of each individual modification in the peptide. Two examples of MS/MS-spectra are given in figure 3. From the data presented in figure 3, it appears that the formaldehyde-glycine attachments are not resistant to fragmentation. This fragmentation started with the loss of a part of the formaldehyde-glycine adduct form the peptide backbone, followed by the fragmentation of the backbone. Fortunately, a small mass tag of 12 Da was left to the modified residue and enabled the identification of the modified sites possible. When two formaldehyde-glycine adducts were attached to tyrosine or arginine residues (resulting in mass increments of 174 and 198, respectively), the MS/MS-analyses demonstrated a residual mass tag of 24 Da. All peptides that could be sequenced by MS/MS corresponded to the assignment based on MS-data. Hence, we assume that the assignments are correct for (most of) the peptides, although this could not be verified by MS/MS-analyses for all peptides. Without MS/MS-analysis, the assignment of a particular modification is not possible when the peptide contains several reactive residues. In that case, we prefer to ascribe the modification to most reactive residues in that peptide, such as arginine and tyrosine. The relative reactivity of amino acid residues was established in previous work (20).

The results of these MS-analyses are summarized in table 5, representing the expected modifications solely based on the reactivity, and the observed modifications of individual amino acid residues. Formaldehyde-induced modifications were observed on ten residues (Gln^{A5}, Tyr^{A14}, Gln^{A15}, Asn^{A18}, Tyr^{A19}, Asn^{A21}, Tyr^{B16}, Arg^{B22}, Tyr^{B26} and Lys^{B29}). On the

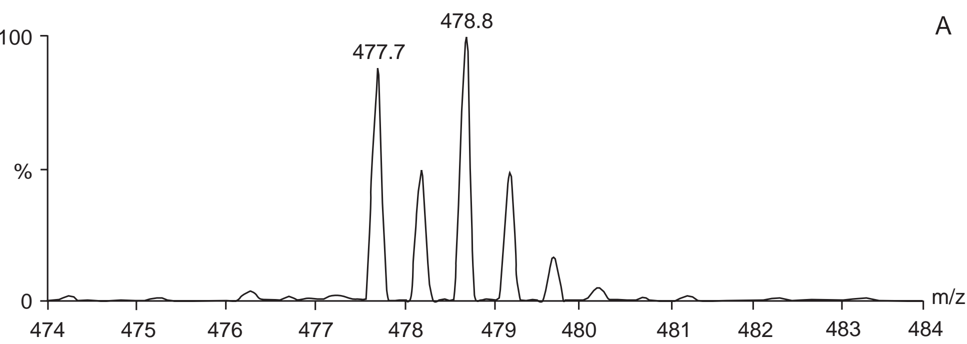
Table 4. Insulin-derived peptides containing formaldehyde/glycine-modified amino acid residues.

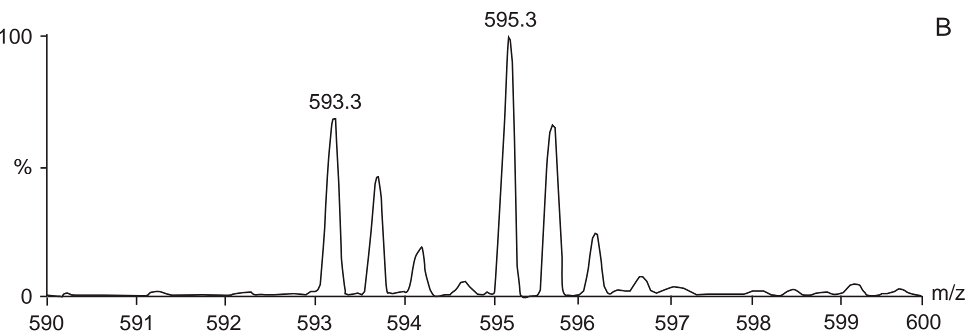
MH+ (DA)	Relative intensity	# CH_2O incorporated	Assigned peptide sequence	Fragment MS/MS	Verified by
954.4	4000	1	AL(Y+87)LVCGE	B14 - B21	yes
1019.6	177	1	SHLVEAL(Y+87)[a)]	B9 – B16	no
1032.4	1230	1	CCASVCSL(Y+87)[a)]	A6 - A14	no
1041.6	3530	2	AL(Y+174)LVCGE	B14 - B21	yes
1119.6	1500	2	CCASVCSL(Y+174)[a)]	A6 - A14	no
1173.6	175	1	RGFF(Y+87)TPKA	B22 - B30	no
1185.4	4090	2	(R+99)GFFYTPKA	B22 - B30	yes
1197.4	2020	3	(R+99)GFF(Y+12)TPKA [b)] (R+24)GFF(Y+87)TPKA [b)]	B22 - B30	yes
1272.1	2700	3	(R+99)GFF(Y+87)TPKA	B22 - B30	no
1284.4	1490	4	(R+99)GFF(Y+99)TPKA [b)] (R+111)GFF(Y+87)TPKA[b)] (R+198)GFFYTPKA	B22-30	yes
1296.4	2370	5	(R+198)GFF(Y+12)TPKA [b)] (R+111)GFF(Y+87)TPKA [b)]	B22 - B30	yes
1371.1	418	5	(R+198)GFF(Y+87)TPKA	B22 - B30	no
1458.1	418	6	(R+198)GFF(Y+174)TPKA	B22 - B30	no
1464.1	533	1	NYCN-AL(Y+87)LVCGE N(Y+87)CN-ALYLVCGE	A18 - A21 and B14 - B21	no
1522.4	1880	1	unknown	unknown	no
1542.1	610	1	unknown	unknown	no
1551.4	1790	2	NYCN-AL(Y+174)LVCGE[c)] N(Y+174)CN-ALYLVCGE N(Y+87)CN-AL(Y+87)LVCGE	A18 - A21 and B14 - B21	no
1600.4	3410	1	unknown	unknown	no
1659.1	910	2	unknown	unknown	no
1679.6	212	2	unknown	unknown	no
1687.6	842	2	unknown	unknown	no
2106.4	552	3	GIVEQCCASVCSL(Y+174)(Q+87)LE, GIVE(Q+87)CCASVCSL(Y+87)(Q+87)LE, GIVEQ(+87)CCASVCSLY(+174)QLE	A1 - A17	no

[a)] Wrong cleavages of insulin by proteinase Glu-C.

[b)] Peptides contain an intrachain cross-link.

[c)] Incomplete reduction of disulfide bridges.





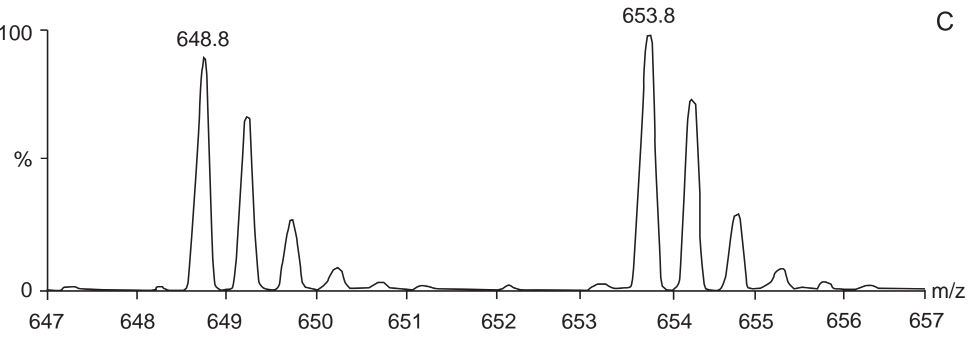


Figure 2. Mass spectra of three formaldehyde-modified peptides (A - C). These three peptides were observed as double protonated ions (MH_2^{2+}), corresponding to MH^+=95.4 (A), 1185.4 (B) and 1296.4 (C). The distance between peptides pairs is a result of the number of incorporated formaldehyde molecules. In these peptides, one, two and five formaldehyde molecules were incorporated, respectively.

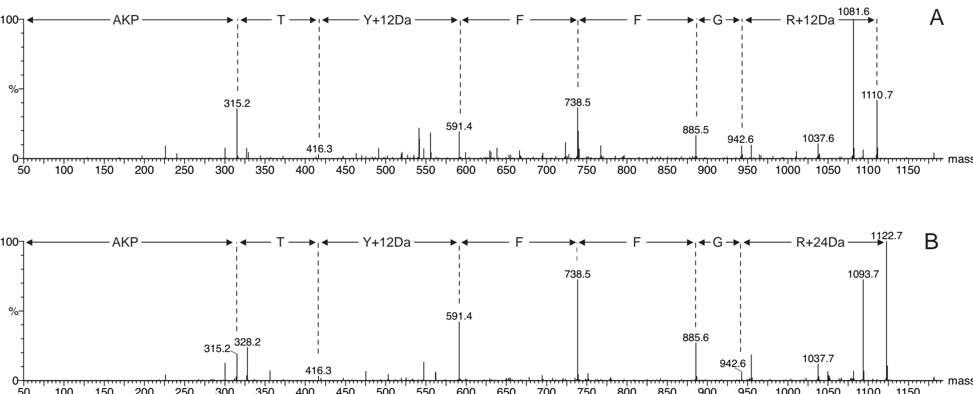


Figure 3. MS/MS-analysis of two peptide fragments of insulin containing formaldehyde induced modifications. Spectrum A is obtained from the peptide (RGFFYTPKA; B22-30) with a formaldehyde-glycine adduct on the arginine (B22) and on the tyrosine (B26) residue, and dominated by a y-ions serie. Spectrum B is acquired from peptide (B22-B30) with probably an intramolecular cross-link between the tyrosine (B26) and lysine (B29) residue and two formaldehyde-glycine adducts on the arginine residue.

other hand, there were no indications that six other residues were modified (Gly^{A1}, Phe^{B1}, Asn^{B3}, Gln^{B4}, His^{B5} and His^{B10}) although these type of residues can react formaldehyde (20). Especially the conversion of N-terminal amino acids was expected, but peptides with modified N-terminal residues could not be found. Instead, non-modified N-terminal peptides were present.

Accessibility of N-terminal residues

No imidazolidinone adducts were observed on the N-terminal amino groups as a result of formaldehyde treatment. This was unexpected, because in general imidazolidinone adducts are rapidly formed by the reaction of formaldehyde with N-terminal amino groups (20-23). Therefore, the reactivity of N-terminal amino groups in insulin for formaldehyde was studied in more detail. Insulin was treated with formaldehyde (CH_2O or CD_2O) and $NaCNBH_3$ to alter the primary amines of insulin into dimethylated amine groups. Indeed, LC-MS analyses revealed masses from N-terminal insulin fragments of the A-chain and the B-chain with a dimethylated glycine (A1) or a dimethylated phenylalanine (B1) residue (Table 6). Thus, formaldehyde reacts with the primary amine groups of N-terminal residues. It is still surprising that no imidazolidinone adducts were formed during the reaction with formaldehyde and glycine. We assume that the second step in the reaction, imidazolidinone formation, did not occur in the A-chain and B-chain of insulin as a result of poor access to the residues, isoleucine (A2) and valine (B2). Indeed, according to the crystal structure, these amino acids are completely buried by surrounding residues (29, 30).

In addition, modified insulin fragments B22-B29 and B22-B30 were observed that have a dimethylated lysine residue (B29) as a result of the reaction with formaldehyde and

Table 5. Formaldehyde-reactive residues in insulin.

Residue[a)]	Position	Accessibility[b)] (%)	Expected (ΔM)	Observed (ΔM)	Verified by MS/MS	Probability[g)]
Glycine	A1	55.3	12/99	-[d)]	-[f)]	high
Glutamine	A5	48.2[c)]	87	87	no	low
Tyrosine	A14	82.7[c)]	87/174	87/174	no	high
Glutamine	A15	45.6[c)]	87	87	no	low
Asparagine	A18	65.4	87	87	no	low
Tyrosine	A19	20.1	87/174	87/174	no	low
Asparagine	A21	90.1	87	87	no	low
Phenylalanine	B1	59.3	12/99	-	-	high
Asparagine	B3	77.1[c)]	87	-	-	low
Glutamine	B4	61.4[c)]	87	-	-	low
Histidine	B5	56.6	87	-	-	low
Histidine	B10	76.5	87	-	-	low
Tyrosine	B16	73.0	87/174	87/174	yes	high
Arginine	B22	53.0	99/198	99/198	yes	high
Tyrosine	B26	28.0	87/174	87/174	yes	high
Lysine	B29	96.6	12	12[e)]	yes	high

[a)] Possible formaldehyde/glycine-reactive amino acid residues in bovine insulin are selected based on previous described study (20).
[b)] The accessibility of each amino acid residue was calculated from the crystal structure of bovine insulin (29) using a described method of Fraczkiewicz and Werner (30). The accessible surface area varied from 0 % for completely buried residues to 100% for surface residues.
[c)] The accessibility of these residues was taken from human insulin (33), because they were not observed in the crystal structure as entire structural entities.
[d)] - = not detected.
[e)] Lysine residues caused intramolecular cross-links with other residues resulting in a mass increase of 12 Da.
[f)] - = not possible.
[g)] The probability that formaldehyde and glycine has modified a particular residue was given based on the reactivity of each type of amino acid, as was earlier shown (20), and the accessibility of each residue.

Table 6. Insulin-derived peptides containing dimethylated amine groups.[a)]

Peptide sequence	MH^+ (Da)	Fragment	Verified by MS/MS
RGFFYTP(K+28)[a)]	1043.6	B^{22} - B^{30}	yes
RGFFYTP(K+28)A	1114.5	B^{22} - B^{30}	yes
(F+28)VNQHLCGSHLVE	1510.9	B^{1} - B^{30}	yes
(G+28)IVEQCCASVCSLYQ[b)]	1628.8	A^{1} – A^{15}	no

[a)] The reaction of formaldehyde and $NaCNBH_3$ resulted in the conversion of primary amines of lysine and N-terminal residues and into dimethylated groups.
[b)] Improper cleavages of insulin by proteinase Glu-C.

$NaCNBH_3$. The fragment B22-B29, which missed the C-terminal alanine residue (B30), probably originates form a degradation product with the mass of 5659 Da, observed during the MS analysis of untreated insulin. The mass difference between insulin and the degradation product is equal to the mass of an alanine residue.

Identification and prevention of intramolecular cross-links

Intramolecular cross-links after the reaction of formaldehyde and glycine with insulin were expected between the lysine (B29) residue and other reactive residues, e.g. arginine (B22) and tyrosine (B26). Insulin peptides with ion masses of 1197.4, 1284.4 and 1296.4 Da could be ascribed to peptides with an intramolecular cross-link (Table 4). The MS and MS/MS-analyses of mixtures of CH_2O/glycine-treated and CD_2O/glycine-treated insulin strongly suggest the presence of insulin-derived peptides with an intramolecular cross-link between tyrosine (B26) and the lysine (B29) residues of the B-fragment, or between the arginine (B22) and lysine (B29) residues.

The MS-measurement revealed the number of incorporated formaldehyde molecules, whereas MS/MS-analysis showed the peptide sequence (RGFFYTPKA), demonstrating that the modifications had occurred on arginine and tyrosine residues. Unfortunately, the results did not reveal between which residues the intramolecular cross-link was located (Table 4). For example, two possibilities can explain the mass of 1197.4 Da: (i) a glycine-formaldehyde adduct on the arginine residue and a methylene bridge between the lysine and the tyrosine residue, or (ii) two methylene bridges between the lysine and arginine residues and a formaldehyde/glycine adduct attached to the tyrosine residue. Both fragments will give the same MS/MS-spectrum as a result of the rapid dissociation of formaldehyde-glycine attachments prior to peptide backbone fragmentation. According to the crystal structure of bovine insulin (29), the distance between the reactive side chains of lysine (B29) and tyrosine (B26) is below the 6 Å, whereas the distance between the lysine (B29) and an arginine (B22) residue is more than 14 Å. Therefore, we assume that the first possibility, a cross-link between lysine and tyrosine, occurred preferably.

The formation of intramolecular cross-links could be prevented by the dimethylations of the lysine (B29) residue. In the previous section, we described that this reaction occurred in insulin by the treatment with formaldehyde and $NaCNBH_3$. The reaction of insulin and dimethylated insulin with formaldehyde and glycine yielded comparable products, except for the intramolecular cross-links (data not shown). Dimethylation of lysine residues can be utilized to improve the analysis of external cross-link reactions to a protein, e.g. the formation of formaldehyde-glycine adducts. Especially, many internal cross-links in large proteins are a drawback, because they are difficult to be digested by proteases, as was observed for diphtheria toxoid (data not shown).

Conclusions

This study demonstrates a great diversity of chemical modifications in the model protein insulin caused by the formaldehyde and glycine treatment. A mixture of formaldehyde and glycine are used of the detoxification of several vaccine antigens, e.g. diphtheria toxin. The data obtained from formaldehyde-treated insulin were consistent with the earlier study with model peptides (20). We have provided a practical method to identify the site and nature of each particular formaldehyde-induced modification in proteins. The investigation revealed the modification of ten amino acid residues in bovine insulin. The approach may be useful to study the complex modifications of larger proteins, such as the formaldehyde treatment of bacterial toxins for preparation of vaccines. The detoxification process of the bacterial toxins is of utmost importance for the antigenicity, immunogenicity and residual toxicity of the resulting toxoid (1, 31, 32). Detailed knowledge about the chemical modifications in these antigens can help to gain a better insight into the relationship between the structure of the antigens and the safety and efficacy of the corresponding vaccines.

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Chapter 7

Identification of formaldehyde-induced modifications in proteins: reactions with diphtheria toxin

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Abstract

Diphtheria toxoid, the principle component of diphtheria vaccines, is prepared by inactivating diphtheria toxin with formaldehyde and glycine. The treatment introduces intramolecular cross-links and intermolecular formaldehyde/glycine adducts in diphtheria toxin. The purpose of the present study was to elucidate the nature and location of formaldehyde-induced modifications at two functional sites of diphtheria toxin: the NAD^+-binding cavity and the receptor-binding site. Therefore, diphtheria toxin was chemically modified using five different reactions: (***1***) dimethylation by formaldehyde and $NaCNBH_3$, (***2***) acetylation by acetic acid N-hydroxy succinimide ester, (***3***) formaldehyde treatment, (***4***) the standard detoxification by formaldehyde and glycine, and (***5***) dimethylation followed by formaldehyde and glycine treatment. The modifications in these experimental diphtheria toxoids were studied by SDS-PAGE, primary amino group determinations (TNBS assay), and/or by liquid chromatography-mass spectrometry (LC-MS) analysis of enzymatically cleaved toxoids. LC-MS analysis confirmed that all but one lysine residue in diphtheria toxin were dimethylated by the reaction with formaldehyde and $NaCNBH_3$ (reaction ***1***). According to the TNBS assay, four amino groups per toxin molecule were on average not dimethylated. The reaction of formaldehyde with lysine residues is the first step in the formation of a stable intramolecular methylene bridge. Thus, the formation of intramolecular cross-links only depends on the close proximity of a second reactive residue. Indeed, LC-MS analysis revealed nine intramolecular cross-links between lysine and a nearby reactive residue in formaldehyde-treated toxin (reaction ***3***). Two masses were ascribed to peptide fragments with an intramolecular cross-link originating from the NAD^+-binding cavity, and two masses for the receptor-binding site. It was assumed that formaldehyde-glycine adducts are connected mainly to the reactive residues at the surface of the protein. As a simplified model, acetylation of lysine residues of diphtheria toxin was investigated (reaction ***2***). LC-MS analysis demonstrated that buried residues are less reactive to the acetic acid N-hydroxy succinimide ester than the residues at the surface of the toxin. This result indicates that formaldehyde-glycine adducts will be only be attached to the more accessible residues. The TNBS assay also demonstrated partial modification of lysine residues (36% unmodified). Finally, the presence of formaldehyde-glycine attachments was studied at the NAD^+-binding cavity and the receptor-binding site (reaction ***5***). Five peptide fragments with formaldehyde-glycine modifications were observed from the NAD^+-binding cavity, and three fragments from the receptor-binding site. In conclusion, the functional sites of diphtheria toxin are affected by the formaldehyde and glycine treatment.

Introduction

Diphtheria toxoid-containing vaccines are included in many national immunization programs. Vaccination has drastically reduced the incidence (and severeness) of diphtheria. Diphtheria vaccines are prepared from diphtheria toxin, which causes the clinical manifestations of the disease. In the 1920, Ramon and Glenny developed independently a successful method for the inactivation of diphtheria toxin, i.e. treatment with formaldehyde (1, 2). The current production process of diphtheria vaccines is essentially based on their method.

In general, the reactions of formaldehyde with amino acid residues are rather well understood. Extensive model studies have revealed the reactivity of amino acid residues and the nature of the modifications (3-8). The reaction of formaldehyde with protein starts with the formation of reversible methylol adducts on amino groups. The methylol groups partially dehydrate, yielding labile Schiff-bases, which can form cross-links with several amino acid residues. The formaldehyde treatment has a great effect on the toxicity, antigenicity and immunogenicity of diphtheria toxin (9, 10). Formaldehyde converts diphtheria toxin into a non-toxic product, called diphtheria toxoid (9), probably by destroying active sites in the molecule, e.g. the NAD^+-binding cavity and the receptor-binding site. However, the exact location and the nature of the modifications at the functional sites are unknown. Furthermore, detoxification causes complete or partial loss of epitopes, as demonstrated with anti-diphtheria toxin monoclonal antibodies (10). In spite of this, the toxoid remains very immunogenic and induces a protecting immune response by the generation of toxin-neutralizing antibodies.

The purpose of this study was to elucidate the chemical modifications in diphtheria toxin as a result of the detoxification. Because mapping of all structural modifications in the entire molecule is very laborious, attention was focused on two functional areas, i.e., the NAD^+-binding cavity and the receptor-binding site. The NAD^+-binding groove is located in the catalytic domain of diphtheria toxin that transfers the ADP-ribose moiety of NAD^+ to elongation factor-2 (EF-2) (11). The modification of EF-2 irreversibly inhibits the protein synthesis in the host cell leading to cell death. Three short peptide sequences in diphtheria toxin form the NAD^+-binding cavity, a loop from the residues 17 – 23, a β-strand followed by an α-helix from residues 50 – 67, and a β-strand from residues 147 – 150 (Figure 1A). The participation of amino acid residues His 21, Tyr 54, Tyr 65 and Glu 148 for the binding of NAD^+ has been described in the literature (12, 13). Another important area in the toxin molecule is the receptor-binding site, which is formed by a loop of amino acid residues between 511 – 530. This part of the receptor domain binds to the heparin-binding epidermal growth factor-like precursor (14, 15). The residues Tyr 514, Lys 516 Val 523, Asn 524, Lys 526 and Phe 530 participate in binding to the host cell receptor (14). The crystal structure of a receptor-bound diphtheria toxin complex is known (16) (Figure 1B). Both the NAD^+-binding cavity and the receptor-binding site contain formaldehyde reactive residues.

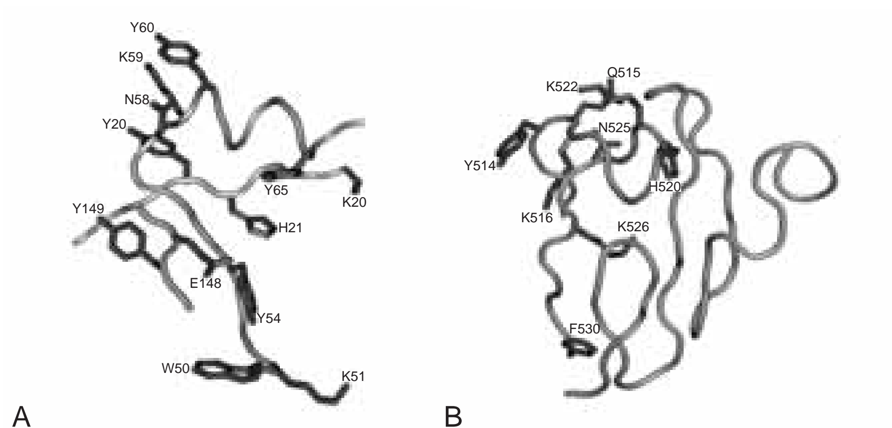

Figure 1. Images that represent two functional sites in diphtheria toxin: the NAD^+-binding cavity (A) and the receptor-binding loop (B). Picture A is based on the crystal structure of diphtheria toxin, and picture B on the crystal structure of the complex between diphtheria toxin and a fragment of cell-surface receptor (HB-EGF). Several side chains of amino acids of diphtheria toxin are represented because of their potential reactivity with formaldehyde or their participation in the protein function.

Materials and methods

Chemicals

Formaldehyde (37%), formic acid (99%), formamide, glycine, potassium dihydrogen phosphate ($KH_2PO_4{\cdot}3H_2O$) and dipotassium hydrogen phosphate ($K_2HPO_4{\cdot}3H_2O$) were purchased from Merck (Amsterdam, The Netherlands). Formaldehyde-D_2 (CD_2O) was supplied by C/D/N Isotopes Inc. (Utrecht, The Netherlands). Acetic acid N-hydroxy succinimide ester, DL-dithiotreitol (DTT) and sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma (Zwijndrecht, The Netherlands). Chymotrypsin, endoproteinase Glu-C, trypsin and subtilisin were bought from Roche Applied Science (Almere, The Netherlands).

Chemical treatment of diphtheria toxin

Prior to reactions, diphtheria toxin (NVI, The Netherlands) was extensively against 10 mM potassium phosphate pH 7.2 (MWCO 10 kDa; CelluSept T3; Membrane Filtration Products, Inc; USA). Furthermore, formaldehyde (CH_2O), deuterium-labeled formaldehyde (CD_2O), glycine and $NaCNBH_3$ were dissolved in water to a concentration of 1.0 M. Acetic acid N-hydroxy succinimide ester was dissolved in formamide up to a final concentration of 0.8 M. Five reactions with diphtheria toxin (3mg/ml) were performed: (***1***) diphtheria toxin with formaldehyde and $NaCNBH_3$, (***2***) diphtheria toxin with acetic acid N-hydroxy succinimide ester, (***3***) diphtheria toxin with formaldehyde (CH_2O or CD_2O), (***4***) diphtheria toxin with formaldehyde (CH_2O or CD_2O) and glycine, and (***5***) diphtheria toxin with formaldehyde

(CH_2O) and $NaCNBH_3$ followed by the reaction with formaldehyde (CH_2O or CD_2O) and glycine. The composition and the reaction conditions for reaction ***4*** are most comparable to those used for vaccine preparation (10). The compositions of the reaction mixtures are given in table 1. For reaction ***1***, formaldehyde (CH_2O) and $NaCNBH_3$ were added to diphtheria toxin. The final concentrations of formaldehyde and $NaCNBH_3$ were 80 mM, and of diphtheria toxin 1.9 mg/ml. After mixing, the solution was incubated for 24 h at 35 °C. For reaction ***2***, N-hydroxy succinimide ester was added to diphtheria toxin. The final concentration of N-hydroxy succinimide ester was 13.3 mM. The mixture was incubated for 24 h at 35 °C. For reaction ***3***, diphtheria toxin was treated with 80 mM formaldehyde (CH_2O) or deuterium-labeled formaldehyde (CD_2O). Both mixtures were incubated for 1 week at 35 °C. For reaction ***4***, diphtheria toxin was treated with formaldehyde (CH_2O or CD_2O) and glycine. The final concentrations of formaldehyde and glycine were 80 mM. The solutions were incubated for 1 week at 35 °C. For reaction ***5***, 3.2 ml of the dialysed product of reaction ***1*** was subsequently incubated for 1 week at 35 °C with formaldehyde (CH_2O or CD_2O) and glycine. The final concentrations of formaldehyde and glycine were 80 mM and of diphtheria toxin 1.2 mg/ml. After each reaction, the samples were extensively dialysed against 10 mM potassium phosphate pH 7.2 (MWCO 10 kDa). For reactions ***3***, ***4*** and ***5***, after incubation and dialysis, equal volumes of the CH_2O-treated and CD_2O-treated samples were mixed. Finally, all samples were stored at 4 °C prior to the analyses.

Table 1. The composition of reaction mixtures.

		Reaction step 1				Reaction step 2	
Mixture[a)]	Diphtheria toxin[b)] toxin[b)]	Formaldehyde	Acetic acid ester	NaCNBH4	glycine	Formaldehyde	glycine
	(3 mg/ml)	(1M)	(0.8M)	(1M)	(1M)	(1M)	(1M)
1	3.2 ml	0.4 ml	-	0.4 ml	-	-	-
2	3.2 ml	-	83 µl		-	-	-
3	3.2 ml	0.4 ml	-	-	-	-	-
4	3.2 ml	0.4 ml	-	-	0.4 ml	-	-
5	3.2 ml	0.4 ml	-	0.4 ml	-	0.4 ml	0.4 ml

[a)] 10 mM potassium phosphate in water (pH 7.2.) was added to obtain a final volume of 5 ml.

[b)] The antigenicity of diphtheria toxin at this concentration (3 mg/ml) was 900 Lf/ml.

SDS-PAGE

SDS-PAGE was performed under reducing conditions, essentially as described by Sambrook et al. (17). Protein samples were prepared by mixing 2 µg of the toxoid in the 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 for 10 min to denature the protein and to reduce disulfide bridges. The samples were loaded onto 10 % SDS-PAGE gels and electrophoretically separated. Molecular weight reference (broad range; Bio-Rad) was used for calibration. Protein bands were visualized by using

Coomassie brilliant blue. The gels were scanned and the intensity of protein bands was quantified by using the program Phoretix 1D quantifier (Phoretix International, UK).

TNBS assay

Primary amino group contents were determined using a colorimetric assay with 2,4,6-trinitrobenzenesulphonic acid (TNBS) (18).

Digestion by proteases

Diphtheria toxoids, obtained from reactions ***1*** and ***2***, were individually digested by the proteases chymotrypsin, endoproteinase Glu-C, trypsin and subtilisin. Diphtheria toxoids from reactions ***3*** and ***5*** were only digested by chymotrypsin. To that end, 10 μl of a 1.0 M buffer and 2 μl of 1.0 mg/ml protease were added to 80 μl toxoid. Water was added to a final volume of 100 μl. Each protease had a specific reaction buffer. The buffer for chymotrypsin was 1 M Tris-HCl pH 8.5 and 0.1 M $CaCl_2$; for proteinase Glu-C 1 M NH_4HCO_3 pH 8.5; for trypsin 1 M Tris-HCl pH 8.5; and for subtilisin 1 M Tris-HCl pH 8.8. Samples treated with, endoproteinase Glu-C, and trypsin were incubated for 24 h at 37 °C. Samples with subtilisin were incubated for 4 h at 37 °C. To reduce disulfide bonds, 1 μl of 0.1 M DTT was added after digestion and the samples were incubated for 1 h at 37 °C. Subsequently, the samples were stored at –20 °C before LC-MS analysis.

LC-MS

Protein digests were analyzed by nano-scale reversed-phase liquid chromatography electrospray mass spectrometry, essentially as previously described by Meiring et al. (19). The analysis was performed on two different mass spectrometers: a ThermoElectron LCQ™ Classic quadrupoe ion trap (San Jose, CA, USA) for the digests of reaction products ***1*** and ***2***, and a Waters Q-TOF Ultima API (Manchester, England) for the digests of reaction products **3** and **5**. The digests of reaction products ***1*** and ***2*** were diluted in water containing 5% (v/v) DMSO and 5% (v/v) formic acid to a concentration corresponding to 1.0 μM of the original protein concentration. An injection volume of 10 μl was used for analysis. Analytes were trapped on a 15 mm long x 100 μm inner diameter trapping column with Aqua C18 (5 μm; Phenomenex) at a flow rate of 3 μl/min of 100% solvent A (0.1 M acetic acid in water) as eluent for 10 min. Then, analytes were separated by reversed-phase chromatography by using a 25 cm long x 50 μm inner diameter. analytical column with Pepmap C18 (5 μm; Dionex) at a flow rate of 125 nl/min. A linear gradient was started with 5 % solvent B (0.1 M acetic acid in acetonitrile) to 60 % solvent B in 55 min. After the run, the columns were equilibrated in 100% solvent A for 10 min of 125 nl/min. The digested peptides were measured by data-dependent scanning comprising a MS-scan (m/z 350 – 2000) followed by collision-induced dissociation of the most abundant ion in the MS spectrum. The data were evaluated by using the TurboSequest software (ThermoElectron). The digests of reaction products ***3*** and ***5*** were diluted with water containing 5% (v/v)

DMSO and 5% (v/v) formic acid to a concentration corresponding to 2.5 nM of the original protein concentration. An injection volume of 10 μl was used for analysis. The same separation procedure was performed as described above to determine the masses of the digested peptides. Peptides containing formaldehyde modifications typically appeared as mass spectral doublets as a result of the use of "light" (CH_2O) and "heavy" (CD_2O) formaldehyde. The modified residues and the peptide sequence were assigned based upon the observed mass and the number of incorporated formaldehyde molecules.

Results

Dimethylation of lysine residues

The modifications in diphtheria toxoid after formaldehyde and glycine treatment consist of intramolecular cross-links and formaldehyde-glycine attachments. The intramolecular cross-links occur between a lysine residue and susceptible amino acid residues, including arginine, asparagine, glutamine, histidine, tryptophan and tyrosine (8). To determine the accessibility of each individual lysine residue for formaldehyde, diphtheria toxin was treated with formaldehyde and $NaCNBH_3$ (reaction ***1***). In this reaction primary amino groups of lysine and N-terminal residues are converted to dimethylated structures with a mass increment of 28 Da (Scheme 1A) (8, 20). The TNBS assay showed a drastic reduction (>95%) of the number of primary amino groups in diphtheria toxin after formaldehyde/$NaCNBH_3$ treatment, which suggests that most lysine residues were modified (Figure 2). Furthermore, SDS-PAGE showed protein bands with slightly increased masses as a result of the dimethylation (Figure 3). Finally, the modified lysine residues were detected by LC-MS analyses of toxoid, which was individually digested with chymotrypsin, endoproteinase Glu-C, trypsin, or subtilisin. Eighty-eight percent of the primary sequence of diphtheria toxin, including the NAD^+ plus the receptor binding site, was detected by LC-

(A)

$$3\ R{-}NH_2 + 6\ H_2C{=}O + 2\ NaCNBH_3 \longrightarrow 3\ R{-}N(CH_3)_2 + 2\ HCN + 2\ NaH_2BO_3$$

(ΔM=+28)

(B)

$$R{-}NH_2 + H_3C{-}C({=}O){-}O{-}N(\text{succinimide}) \longrightarrow R{-}NH{-}C({=}O){-}CH_3 + HO{-}N(\text{succinimide})$$

(ΔM=+42)

Scheme 1. Methylation (A) and acetylation (B) of lysine residues in proteins.

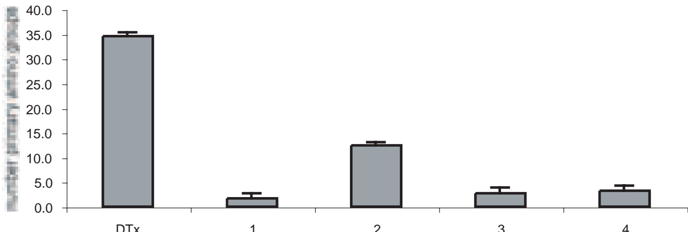

Figure 2. The number of primary amino groups in diphtheria toxin (DTx) and several toxoids (1 - 4) (mean ± S.D; n = 6). The toxoids were prepared by reaction ***1***, reaction ***2***, reaction ***3***, and reaction ***4*** (see Materials & Methods for details).

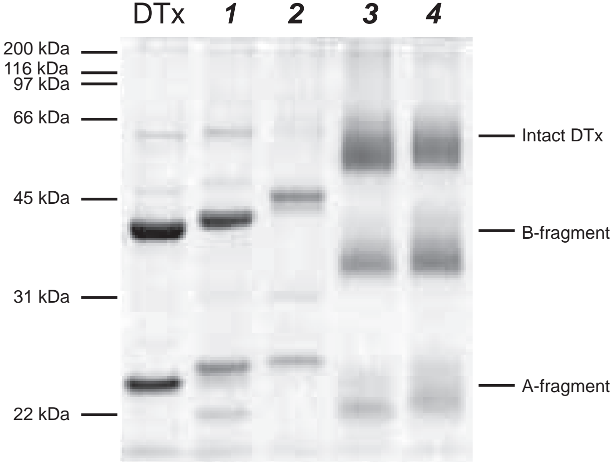

Figure 3. SDS-PAGE diphtheria toxin (DTx) and several toxoids (1 - 4). The toxoids were prepared by reaction ***1***, reaction ***2***, reaction ***3***, and reaction ***4*** (see Materials & Methods for details).

MS. Dimethylation was observed for all but one lysine residue in diphtheria toxin. Only dimethylation of the residue Lys 456 could not be confirmed. Also, the unmodified residue Lys 456 was not observed. The accessible surface area of this residue is 9.1%, as calculated according to Fraczkiewicz and Braun (21). Also from the crystal structure of diphtheria toxin (22) it becomes apparent that residue Lys 456 is very inaccessible as a result of steric hindrance by surrounding residues. However, other "inaccessible" lysine residues, e.g. Lys 103 with an accessible surface of 2.6%, were modified. Thus, we assume that the residue Lys 456 residue can also react with formaldehyde. Although the TNBS assay indicates the presence of a few primary amino groups in the diphtheria toxoid (from reaction **1**), no unmodified lysine residues were observed by LC-MS.

Acetylation of lysine residues

In our previous study with model peptides, we demonstrated that under conditions commonly used for vaccine preparation, formaldehyde primarily reacts with glycine and the formaldehyde-glycine adducts are then attached to several receptive amino acid residues (8). We assumed that these adducts are connected mainly to the reactive residues at the surface of the protein. Therefore as a simplified model, the attachment of acetyl groups to lysine residues of diphtheria toxin was investigated (reaction ***2***; Scheme 1B). The acetylation by acetic acid N-hydroxy succinimide ester was intended to mimic the attachment of formaldehyde-glycine adduct. The reaction caused a mass increase of diphtheria toxin, as was visualized by SDS-PAGE (Figure 3; lane ***2***) The mass increase was more than for the dimethylated toxin. Furthermore, a strong reduction of the number of primary amino groups (64%) was observed (Figure 2), although not as high as with dimethylation (96%; reaction ***1***). Apparently, the acetic acid N-hydroxy succinimide ester reacted much slower with buried lysine residues than the more exposed lysine residues (reaction ***2***).

LC-MS was used to demonstrate the extent of acetylation. Therefore, the toxoid obtained from reaction ***2*** was individually digested with chymotrypsin, endoproteinase Glu-C, trypsin, and subtilisin. Peptide fragments were detected with acetylated (mass increment of 42 Da) and non-acetylated lysine residues. Most lysine residues (27 out of 39) were completely acetylated. However, twelve lysine residues only partially reacted with the acetic acid ester, probably as a result of reduced reactivity (Table 2). In general, the partially modified residues have small accessible surface areas as a result of shielding by surrounding residues (Table 2). However, there are exceptions to this rule: according to the crystal

Table 2. Calculated accessibility of partially acetylated lysine residues of diphtheria toxin (reaction ***2***).

Lysine residue no.	Calculated accessible surface[a)] %
51	28
59	36
103	3
157	64
216	5
229	18
242	39
244	53
264	49
419	64
440	21
522	72

[a)] The accessibility of each amino acid residue was calculated from the crystal structure of diphtheria toxin using a described method of Fraczkiewicz and Werner (21). The accessibility varied from 0 % for completely buried residues to 100% for surface residues. The accessible surface area of fully acetylated lysine residues were on average 56 ± 18% (mean ± S.D.), whereas of the partially acetylated residues 38 ± 22%.

structure, residues Lys 90, Lys 172 and Lys 385 are rather inaccessible (accessibility of 6.4%, 15.2% and 12.4%, respectively), but these residues showed 100% conversion.

Formaldehyde-induced cross-links in diphtheria toxin

The treatment of diphtheria toxin with a mixture of formaldehyde and glycine (reaction ***4***) results in a very heterogeneous toxoid. The heterogeneity could be visualized by SDS-PAGE, showing a very diffuse protein band of diphtheria toxoid compared to diphtheria toxin (Figure 3; lane ***4***). In theory, 140 residues in diphtheria toxin can react with formaldehyde. Since many residues are partly converted, diphtheria toxoid will consist of thousands of different reaction products. As a result of the high diversity in modifications, it is very laborious to determine all modified residues in this large protein (58.3 kDa). Therefore, in the present study we have focused on the formaldehyde-induced modifications in two areas of diphtheria toxin, which are important for the toxic action: (i) the NAD^+-binding groove in the catalytic domain, and (ii) the loop in the receptor domain involved in receptor binding.

In reaction ***3***, diphtheria toxin was treated with formaldehyde (CH_2O) or with deuterium-labeled formaldehyde (CD_2O) to introduce mainly intramolecular cross-links in the protein. Intermolecular cross-linking between two toxin molecules hardly occurred, otherwise it was observed by SDS-PAGE (Figure 3; lane ***3***). LC-MS analysis of chymotrypsin-digested mixtures of CH_2O-treated and CD_2O-treated toxin revealed eleven fragments containing an intramolecular cross-link (Table 3). Cross-links can be identified by their typical mass increases (ΔM = +12 or +24 Da) (8).

Besides intramolecular cross-links, the formaldehyde-glycine attachments in diphtheria toxin were investigated. Prior to the formaldehyde and glycine reaction, lysine residues in diphtheria toxin were dimethylated to prevent intramolecular cross-linking (reaction ***5***). LC-MS analysis of the chymotrypsin-digested product revealed 82 peptides with formaldehyde modifications, as was clear from mass spectral doublets by the use of CH_2O and CD_2O. Thirty-six peptide sequences with modifications could be assigned based on the observed masses and the number of incorporated formaldehyde molecules. The assigned peptides cover 40% of the whole diphtheria toxin sequence.

The standard inactivation reaction for vaccine preparation (reaction ***4***), results in intramolecular cross-links, as observed in toxoids from reaction ***3***, and formaldehyde-glycine attachments, found in toxoids from reaction ***5***. The individual modifications at the NAD^+-binding cavity and the receptor-binding site have not been identified in this product (reaction ***4***).

Modifications in the catalytic site

Two toxin fragments with intramolecular cross-links were observed that belong to the NAD^+-binding groove (reaction ***3***). The cross-link is probably formed between the residues Lys 59 and Tyr 60 (peptides 2 and 3; Table 3). The distance of the side chains of both

Table 3. Diphtheria toxin-derived peptides with an intramolecular cross-link.[a)]

Peptide	Sequence	Fragment	Observed Mass
1	GADDVVDSSKSF[b)]	1-12	1238.6 (0.0)[c)]
2	STDNKYDAAGY	51-60	1216.6 (-0.1)
3	DDDWKGFYSTDNKYDAAGY	47- 65	2242.2 (0.7)
4	IKRFGDGASRVVL	124-136	1441.9 (-0.1)
5	IKRFGDGASRVVLSL	124-138	1641.7 (0.2)
6	INNWEQAKAL	150-159	1198.6 (0.0)
7	DVIRDKTKTKIESL	207-220	1669.9 (0.1)
8	NRPAYSPGHKTQPFL	376-390	1611.8 (0.0)
9	HRSSSEKIHSNEISSDSIGVL	492-512	2294.0 (-0.1)
10	GYQKTVDHTKVNSKLSL, GYQKTVDHTKVNSKLSL	513-528	1929.3 (0.7)
11	QKTVDHTKVNSKLSLF	515-530	1857.8 (-0.7)

[a)] Diphtheria toxin-derived peptides were obtained from reaction mixture 3 after the digestion with chymotrypsin (see Materials & Methods for details).
[b)] Intramolecular cross-links were most likely formed between the underlined residues. Both side chains are close to each other (<5Å), according to the crystal structure (22).
[c)] Deviation from the theoretical mass.

residues is about 2 Å (22). However, no cross-links were observed between Lys 59 and two other tyrosine residues, Tyr 20 and Tyr 181, although they are in close proximity of the lysine residue (ca. 4 Å). The masses of the expected cross-linked peptide fragments are presented in table 4. However, we assume that incomplete digestion by chymotrypsin resulted in several overlapping peptides, which are not recognized as such. Furthermore, the applied LC-method might be unsuitable for such large and probably hydrophobic peptide fragments, i.e. extremely long retention times or no elution at all.
Five peptide fragments with formaldehyde-glycine attachments were found, originating from the catalytic cleft (reaction ***5***; Table 5). However, the exact location of the modified residue(s) could not be determined, because all these assigned peptides contain two or more reactive residues. Previous work with synthetic peptides enables us to propose the 'most likely' structure of the modified peptides (Table 5). The tyrosine and arginine residues for

Table 4. Expected peptide fragments from the NAD$^+$-binding groove containing an intermolecular cross-link.[a)]

Sequence	Cross-link between	MH+	Mass observed
STDNKYDAAGY	Lys 59 and Tyr60	1216.5	yes
SSYHGTKPGY STDNKY	Tyr 20 and Lys 59	1834.8	no
STDNKY KEHGPIKNKMSESPNKTVSEEKAKQYL	Tyr 181 and Lys 59	3838.9	no

[a)] The peptides were expected after digestion of diphtheria toxoid, prepared with reaction ***3***.

Table 5. Modified peptide fragments from the NAD+-binding site of diphtheria toxin.[a)]

MH+ (Da)	# CH_2O incorporated	Assigned peptide sequence [c)]	Fragment
874.4 (0.0)[b)]	1	**(H+87)GT(K+28)PGY** [d)] HGT(K+28)PG(Y +87)	21-27
1451.6 (0.2)	1	**DAAG(Y +87)SVDNENPL** DAAGYSVD(N+87)ENPL DAAGYSVDNE(N+87)PL	61-73
1538.6 (0.2)	2	**DAAG(Y +174)SVDNENPL** DAAG(Y +87)SVD(N+87)ENPL DAAG(Y +87)SVDNE(N+87)PL DAAGYSVD(N+87)E(N+87)PL	61-73
1542.8 (0.2)	1	**AEGSSSVE(Y+87)INNW** AEGSSSVEYI(N+87)NW AEGSSSVEYIN(N+87)W AEGSSSVEYINN(W+87)	141-153
1630.0 (0.4)	2	**AEGSSSVE(Y+174)INNW** AEGSSSVE(Y+87)I(N+87)NW AEGSSSVE(Y+87)IN(N+87)W AEGSSSVE(Y+87)INN(W+87) AEGSSSVEYI(N+87)(N+87) W AEGSSSVEYI(N+87)N(W+87) AEGSSSVEYIN(N+87)(W+87)	141-153

a) The product of mixture 5 was digested by chymotrypsin (see Materials & Methods for details).
b) Deviation from the theoretical mass.
c) Lysine residues were dimethylated (ΔM +28 Da) prior to formaldehyde-glycine treatment.
d) Peptides containing the most likely modification(s) based on results obtained with synthetic peptides (8) are indicated in bold.

instance are the most reactive amino acids. The results indicate that formaldehyde-glycine adducts are formed at the residues His 21, Tyr 65 and Tyr 149. Amino acid residues His 21 and Tyr 65 are involved in the binding of NAD^+, whereas Tyr 149 is the adjacent residue of Glu 148 that participates in the binding. In conclusion, the catalytic cleft is affected by intramolecular cross-links and formaldehyde-glycine attachments.

Modifications in the receptor-binding site

A second location for which the effect of formaldehyde treatment was studied in more detail was the receptor-binding site of diphtheria toxin. With LC-MS analyses of the toxoid obtained from reaction ***3***, two masses were found belonging to peptide fragments of the receptor-binding site of diphtheria toxin (peptides 10 and 11; Table 3). The peptides probably contain an intramolecular cross-link between the amino acid residues Lys 522 and Gln 515, or between residues Lys 516 and Tyr 514.

Furthermore, three masses were found that could be ascribed to peptides with formaldehyde-glycine adducts (reaction ***5***; Table 6). The adducts are probably formed at

amino acid residues Tyr 514, His 520 and Asn 524. For residues Tyr 514 and Asn 524 participation in receptor binding has been demonstrated (14). Thus, both the receptor-binding site and the NAD^+-binding site are modified during the detoxification reaction.

Table 6. Modified peptide fragments from the receptor-binding site of diphtheria toxin.[a)]

MH+ (Da)	# CH_2O incorporated	Assigned peptide sequence	Fragment
1669.0 (0.1)[b)]	1	**Q(K+28)TVD(H+87)T(K+28)VNS(K+28)L**[c) d)]	515-527
		Q(K+28)TVDHT(K+28)V(N+87)S(K+28)L	
1889.2 (0.4)	1	**G(Y+87)Q(K+28)TVDHT(K+28)VNS(K+28)L**	513-527
		GYQ(K+28)TVD(H+87)T(K+28)VNS(K+28)L	
		GYQ(K+28)TVDHT(K+28)V(N+87)S(K+28)L	
1976.2 (0.4)	2	**G(Y+174)Q(K+28)TVDHT(K+28)VNS(K+28)L**	513-527
		G(Y+87)Q(K+28)TVD(H+87)T(K+28)VNS(K+28)L	
		G(Y+87)Q(K+28)TVDHT(K+28)V(N+87)S(K+28)L	
		GYQ(K+28)TVD(H+87)T(K+28)V(N+87)S(K+28)L	

[a)] The product of mixture 5 was digested by chymotrypsin (see Materials and Methods and Table 1 for details).
[b)] Deviation from the theoretical mass.
[c)] Lysine residues were dimethylated (ΔM +28 Da) prior to formaldehyde-glycine treatment.
[d)] Peptides containing the most likely modification(s) based on results obtained with synthetic peptides are indicated in bold (8).

Discussion

In the present study, the type and extent of formaldehyde-induced modifications of diphtheria toxin were investigated, with a detailed analysis of the NAD^+-binding groove and the receptor-binding site. In both areas of the toxin molecule, intramolecular cross-links and formaldehyde-glycine attachments were found. The conversion of these sites probably contributes to the inactivation of diphtheria toxin. In principle, all primary amino groups in diphtheria toxin are accessible for formaldehyde as demonstrated by the reaction with formaldehyde and $NaCNBH_3$ (reaction ***1***). It was demonstrated for a few lysine residues that they form intramolecular cross-links (reaction ***3***). The observed intramolecular cross-links (Table 3) are formed between residues that are in close proximity (<5Å). Several other intramolecular cross-links were expected according to the crystal structure. When the toxin is in solution, these cross-links might not be formed because of the high local mobility of the reactive amino acid residues involved in the formation of cross-links. The distance between the residues is probably too large to be effective in cross-linking.

Steric hindrance is probably a major factor contributing to the low or absent reactivity of amino acid residues with formaldehyde-glycine. The effects of steric hindrance were studied in a model reaction of diphtheria toxin with acetic acid N-hydroxy succinimide ester (reaction ***2***). Indeed as was observed, acetylation of buried amino (lysine) groups is in general not quantitative, whereas the more accessible residues were fully converted under the studied conditions. It is however likely that a similar phenomenon will be oberved for

the attachments of formaldehyde and glycine adducts to diphtheria toxin.
The detoxification process changes the antigenicity and immunogenicity of diphtheria toxoid (10). Although we observed many other masses related to modified peptides belonging to other parts in diphtheria toxin than the NAD^+-binding cavity and the receptor-binding loop, their contribution in reducing the toxicity or in changing the antigenicity is unknown. Theoretically, every intramolecular cross-link in the protein may be sufficient to inactivate the toxin. The formaldehyde-induced modifications on or near the immunodominant epitopes of diphtheria toxin are of interest to know, because these might affect the potency of the vaccine. The exact locations of immunodominant epitopes of diphtheria toxin are not yet known. Identification of these epitopes will be subject to future work.
In conclusion, the approach followed in this study is suitable to identify formaldehyde-induced modifications in diphtheria toxoid. The methods described here are suitable for the characterisation of diphtheria toxoids and, probably, also for other formaldehyde-inactivated antigens, including tetanus toxoid, pertussis toxoid and inactivated polio vaccine. The methods can be used in comparability studies, e.g. to support registration of these products after process or formulation improvements. The work demonstrates that with current powerful analytical methods it is possible to approach classical antigens as if they were well-defined biologicals.

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Chapter 8

Immunochemical characterisation of Monoclonal Antibodies for Quality Control of Diphtheria Vaccines

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Abstract

The current potency tests for diphtheria vaccines are an intradermal challenge test in guinea pigs or a serological assay in mice. The purpose of the present study was to select monoclonal antibodies which can be used in an alternative in vitro test to establish the quality of diphtheria toxoids. Fifteen anti-diphtheria toxin monoclonals were mapped by competitive surface plasmon resonance (SPR). The panel of fifteen antibodies could be divided into five groups recognising non-overlapping epitopes. Representative antibodies from each epitope group, Dim 5, Dim 9, Dim 25, Dim 27 and Dim 33, were tested for toxin neutralising activity. Subsequently, they were used to reveal immunodominant epitopes in diphtheria toxin by SPR analysis. Due to instability of immobilised diphtheria toxoid a new immobilisation procedure was developed. Monoclonals Dim 9 and Dim 25 showed tenfold higher toxin neutralising activity than the Dim 33. The toxin neutralising capacities of monoclonals Dim 5 and Dim 27 were in between. In addition, monoclonals Dim 5 and Dim 9 demonstrated substantial inhibition of the binding of mouse antiserum to diphtheria toxin, indicating that these two epitopes are immunodominant. Dim 5 and Dim 9 reduced the binding of the serum to 65% and 45% of original values, respectively. The other three antibodies diminished serum binding maximally 10%. The combination of the five non-overlapping monoclonal antibodies could almost completely block the binding of sera to diphtheria toxin. In conclusion, five anti-diphtheria monoclonal antibodies were selected that are representative for the humoral response induced by immunisation of mice with a diphtheria vaccine. These antibodies could be applied in an immunochemical assay as a possible substitute for the *in vivo* potency test of diphtheria vaccines.

Introduction

Lot release of diphtheria toxoid vaccines requires the assessment of the potency, which is commonly done in an intradermal challenge test in guinea pigs (1). A large number of at least 100 animals is required for one batch to demonstrate that the vaccines induce a protecting immune response. An important drawback of the *in vivo* test is the suffering of the animals due to the challenge with diphtheria toxin. Furthermore, the accuracy and precision of this test is questionable, because the only parameter in this test is the number of surviving guinea pigs. Several producers currently apply a serological method to establish the potency of the vaccine. For this purpose, an *in vitro* toxin neutralisation test (TN) and a toxin binding inhibition (ToBI) assay have been developed and validated (2-4). The neutralisation test is an assay based upon the sensitivity of Vero cells to diphtheria toxin. The neutralising antibody levels can be determined by measuring the inhibition of the cytotoxic action of diphtheria toxin. The ToBI test, a refined ELISA, has shown to be a reliable method to estimate the potency of diphtheria vaccines. The assay correlates with *in vivo* and *in vitro* toxin neutralisation tests. These assays are successful examples of refinement (i.e., less suffering) and reduction of animal use (5), but animals are still necessary. For ethical, economical and practical reasons, the animal use for quality control of vaccines must be reduced, which makes the quest for an alternative highly relevant. To that end, monoclonal antibody based assays are very interesting, because they have the potential to reveal antigenic properties of vaccines that are important for inducing protection.

In vaccine quality control, monoclonal antibodies are often used for the identification and quantitation of antigens. However, they are applied to a lesser degree to investigate the potency of vaccines. A few examples are described in the literature: monoclonals are used for measuring the D antigen content of the inactivated poliomyelitis vaccine (6, 7), for monitoring consistency in production of rabies vaccines (8), and for determining the potency of Hepatitis A and Haemophilus influenzae type b vaccines (9-12). In general, monoclonal antibodies are selected for quality control based upon their specific properties. For example, they recognise a protecting antigenic conformation, have neutralising activity, or bind to a highly conserved epitope on antigens from various strains. Several researchers have described the use of monoclonal antibodies directed against diphtheria toxin (13-17). Their properties have been used to determine structure-function relations and to neutralise diphtheria toxin. However, no studies showed that they are suitable to determine the quality of diphtheria vaccines.

The aim of the present study was to select a panel of monoclonal antibodies that can be applied in an immunochemical assay (ELISA and/or biosensor assay) as a possible substitute for the *in vivo* potency test. The selection criteria were: ((i) the combination of antibodies almost completely inhibits the binding of mouse antiserum against diphtheria toxin, i.e. together they cover most antigenic sites; (ii) the antibodies recognise

immunodominant epitopes or (iii) have high toxin neutralising activity. The study resulted in a restricted collection of monoclonal antibodies that we would recommend for characterising diphtheria toxoids vaccines to assess their quality.

Materials and methods

Chemicals, biochemicals and immunochemicals

N-succinimidyl 3-(2-pyridylthio) propionate (SPDP) was purchased from Pierce. Dimethyl sulphoxide (DMSO), 2,4,6-trinitrobenzene sulfphonic acid (TNBS), 3,3’,5,5’-tetramethyl benzidine (TMB), cystamine, and dithiotreitol (DTT) were obtained from Sigma. Ammonium thiocyanate (NH_4SCN) sodium carbonate, sodium chloride, sodium hydroxide, disodium hydrogen phosphate, sodium dihyhydrogen phosphate, 30% hydrogen peroxide, Tween 80 and sulphuric acid were acquired from Merck. HBS-EP buffer, glycine-HCl pH1.5, ethanolamine and 2-(2-pyridinyldithio) ethaneamine (PDEA), 1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), glycine-HCl, pH 1.5, and rabbit anti-mouse Fc-specific antibody were bought from Biacore AB. Bovine serum albumin (BSA) was from Organon. Diphtheria toxin-containing culture fluid (clarified, concentrated and dialysed) and toxoid were obtained from the production department of the NVI. Monoclonal anti-diphtheria toxin antibodies were obtained from the Laboratory for Vaccine Preventable Diseases of the National Institute of Public Health and the Environment (RIVM). Goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgG, and Ig were purchased form Southern Biotechnology Associates Inc.

Epitope mapping

Epitope mapping of fifteen monoclonal antibodies was performed by using surface plasmon resonance (Biacore 1000). By using an amine coupling kit, Fc-specific antibodies were coupled to the sensor chip (CM5; Biacore), which resulted in a response between 2000 and 3000 resonance units (RU). Subsequently, an anti-diphtheria toxin monoclonal diluted in HBS-EP buffer was captured by the Fc-specific antibodies resulting in a response of 300 – 500 RU. The residual Fc-specific antibodies were blocked by non-specific monoclonal antibody. Diphtheria toxin diluted in HBS-EP buffer (60 μg/ml) was injected until a plateau level was obtained (typically in 2 min, at a flow rate of 5 μl/min). The binding of a second anti-diphtheria toxin monoclonal was analysed after 2 minutes (flow rate of 5 μl/min). The sensor chip was regenerated by the injection of 15 μl of glycine-HCl, pH 1.5. Antibodies with overlapping epitopes were arranged in the same group. From each group, one antibody was chosen for further studies (see Table 1).

Immunisations
Diphtheria toxin, purified diphtheria toxin that was inactivated by formaldehyde and glycine, and diphtheria toxoid that was purified after inactivation were used as antigens for the preparation of vaccines A, B, and C, respectively. The vaccines were prepared as described earlier (17). The protein content in the vaccines A, B, and C were 20 μg/ml, 20 μg/ml, and 60 μg/ml, respectively. Each vaccine was injected in the groin of eight mice (NIH, female, weight 10 – 14 g) receiving 250 μl of the vaccine. After 35 days animals were bled and the blood was individually collected in tubes. To obtain cell-free sera, 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 x g. The supernatants were transferred to new tubes and centrifuged once again. Then, the supernatants were incubated for 45 min at 56 °C to inactivate the complement. To obtain sufficient sera for biosensor analysis, the supernatants from the eight mice were pooled and stored at –20 °C prior to use. The sera form the mice immunised with vaccine A, B, or C were called A, B and C, respectively.
Toxin neutralisation test
The toxin-neutralising capacity of the mouse sera, monoclonal antibodies and combinations of monoclonals was determined in the toxin neutralisation test, essentially as earlier described (17). Prior to this assay, the monoclonal antibodies Dim 5, Dim 9, Dim 25, Dim 27 and Dim 33 were individually diluted to final concentrations of 1730, 660, 530, 600 and 670 nM, respectively. Furthermore, three different combinations were prepared from monoclonal antibodies: (i) mixture 1, monoclonal Dim 5, Dim 9 and Dim 33 with final concentrations of 1730, 660 and 670 nM, respectively; (ii) mixture 2, Dim 25 and Dim 27 in concentrations of 530 and 600 nM, respectively; (iii) mixture 3, monoclonals Dim 5, Dim 9, Dim 25, Dim 27 and Dim 33 in concentrations of 1730, 660, 530, 600 and 670 nM, respectively. Subsequently, toxin neutralisation test was performed by preparing twofold dilution series of three individual sera, five diluted monoclonals and three combinations of monoclonals with complete medium 199 so that each well of the microtitre plate contained 50 μl. Then, 50 μl toxin DT 79/1 with a concentration of 10 ng/ml (0.001 Lf/ml) in complete medium 199 was added to the wells. The plates were incubated for 2 hours at 37 °C. Subsequently, 50 μl complete medium 199 with $5{\cdot}10^5$ Vero cells/ml was added to each well. The plates were covered with a plate sealer and incubated for 6 days at 37 °C and 5% CO_2. The scores (the number of wells containing living cells) of each vaccine was determined by using the microscope. The living cells form an intact monolayer within 6 days. The data is presented as the actual protein concentration of monoclonals or sera that can neutralise 10 ng/ml toxin.

ELISA for determining antibody titres
Anti-dipththeria toxin antibody titres were determined for the classes IgM and IgG, and the subclasses IgG1, IgG2a, IgG2b and IgG3 by an ELISA. Therefore, flat-bottom 96-well ELISA plates (Greiner) were coated overnight at room temperature with 1.6 μg/ml

diphtheria toxin in 0.04 M sodium carbonate, pH 9.6. After the incubation, the plates were washed three times with a washing solution (0.03% (v/v) tween 80 in water). The plates were incubated for 60 min at room temperature with blocking buffer (1% (w/v) BSA in phosphate-buffered saline (PBS); 0.15 M NaCl, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.2) to prevent non-specific binding of serum. The plates were washed and incubated for 120 min at room temperature with 100 µl of serum samples serially diluted with dilution buffer (0.5% BSA and 0.05% (v/v) tween 80 in PBS). The plates were washed and incubated at room temperature for 120 min with 100 µl of a 5,000-fold diluted (sub)class-specific goat anti-mouse Ig conjugate. After the washing, 100 µl of a peroxidase substrate (25 mM TMB and 0.01% H_2O_2 in 0.11 M sodium acetate buffer, pH 5.5) was added to each well, the plates were incubated at room temperature, and the reaction was terminated after 10 min by adding 100 µl of 2 M H_2SO_4 to each well. Finally, the absorbance was recorded at 450 nm with a plate reader (Bio-kinetics reader EL312e, Bio-tec instruments). The antibody titres were expressed as the $\log_{10}$ of the serum dilution giving 50% of the maximum optical density at 450 nm.

ELISA for determining avidity

The avidity of sera to diphtheria toxin was determined by an ELISA method as described above with minor modifications. Briefly, plates were coated with diphtheria toxin, washed, blocked with BSA, and washed. Then, 100-times diluted serum was added to each individual well and the plates were incubated at room temperature for 120 min. Subsequently, the plates were washed, different dilutions of NH_4SCN (ranging 0 – 4 M) were added, and the mixtures were incubated for 30 min resulting in a concentration-dependent dissociation of the antibody-antigen complex. Afterwards, the plates were washed, incubated with Ig-conjugate, washed, and treated with a peroxidase substrate. The absorbance was measured at 450 nm. The avidity data were presented as the NH_4SCN concentrations that resulted in 50% reduction of the maximum optical density at 450 nm.

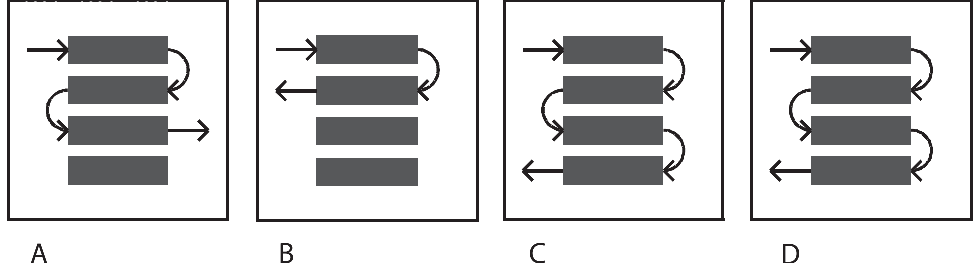
Development of biosensor method

In a preliminary study, diphtheria toxin, when directly bound to a conventional (CM5) biosensor chip, was found to be very labile when applying the described regeneration solutions (18). Therefore, diphtheria toxin was immobilised as ligand to a CM5 sensor chip in each measurement via a thiol coupling and removed afterwards in the regeneration step. To that end, primary thiol groups were incorporated in the dextran matrix of the biosensor (CM5). The carboxyl groups in the dextran surface of the sensor chip were activated by injecting 100 µl of a mixture containing 0.2 M EDC and 0.05 M NHS resulting in reactive succinimide esters. To introduce disulphides groups, 100 µl of cystamine in 0.1 M sodium borate buffer (pH 8.5) was injected. 100 µl of 1.0 M ethanolamine-HCl (pH 8.5) was injected to deactivate possibly non-reacted succinimide esters. Finally, 15 µl of 0.5 M DTT in 100 mM NaOH was used to reduce disulphides and to generate primary thiol groups in

the dextran matrix. Because diphtheria toxin has no primary thiol groups, the toxin was treated with SPDP to introduce them in the protein. SPDP reacts with primary amino groups, i.e. with lysine residues and the N-terminal amino groups in proteins (19). For incorporation of thiol groups in diphtheria toxin, 5 µl of 20 mM SPDP dissolved in DMSO was added to 2 ml of 3 mg/ml diphtheria toxin. After mixing, the solution was incubated for 30 min at room temperature. The thiolated toxin was purified on a desalting column (PD10, Amersham) to remove non-reacted SPDP. After the purification, the primary amino group concentration was determined by a colourimetric assay using TNBS (20). When lysine residues in the toxin were modified to a large extent (>65%), monoclonal antibodies such as Dim 5 and Dim 25 did not recognise the toxin anymore. Therefore, the amount of SPDP attachments was kept low to prevent the destruction of epitopes. On average 2.1 SPDP molecules were randomly incorporated in the toxin as determined by the reduction of the number of primary amino groups. The disulphide bridges are easily reduced by DTT to remove the immobilised toxin from the sensor chip. Subsequently, fresh diphtheria toxin was coupled to the sensor. The method has been used to measure the binding of sera A, B, and C to diphtheria toxin by SPR analysis. No binding of serum from non-immunised mice was observed to immobilised toxin.

Biosensor analysis

To reveal immunodominant epitopes, the binding of mouse antiserum to diphtheria toxin with blocked and non-blocked epitopes were measured by using surface plasmon resonance (Biacore 3000). Each measurement consists of four fundamental steps: (i) immobilisation of diphtheria toxin in flow cells 1-3; (ii) blocking of diphtheria toxin epitopes by monoclonal antibodies in flow cell 1 and 2; (iii) detection of serum binding in all flow cells; and (iv) regeneration to remove bound serum, monoclonal antibodies and immobilised diphtheria toxin (see also Figure 1). In detail, the measurement started with injecting at a flow rate of 5 µl/min 15 µl of 0.5 M DTT in 100 mM NaOH solution for regeneration of



A B C D

Figure 1. Biosensor analysis of anti-diphtheria toxin sera performed in four consecutive steps: (A) immobilisation, (B) blocking, (C) serum binding, and (D) regeneration. Firstly, diphtheria toxin was immobilised via disulphide coupling on sensor surface 1 to 3. Secondly, an antitoxin monoclonal was injected through flow cells 1 and 2 to block an epitope on diphtheria toxin. Thirdly, the binding of mouse antitoxin serum was measured in all four flow cells. Finally, the sensor chip was regenerated to obtain free thiol groups.

free thiol groups, because they form spontaneously disulphides. Then, SPDP-labelled toxin was immobilised in a range of 300 – 1000 RU on the sensor areas of flow cells 1 to 3 (connected in series) by injecting 20 to 40 μl of the 5 – 40 μg/ml toxoid samples in 10 mM NaAc, pH 5.6. Residual primary thiol groups were blocked by injection of 15 μl of 45 mM PDEA through flow cells 1 to 4. Monoclonal antibody was diluted 100-fold in HBS-EP buffer and 50 μl of the solution was injected at a flow rate of 2 μl/min through flow cells 1 and 2. Sera from diphtheria toxoid vaccinated mice was diluted 10 times in HBS-EP buffer and 50 μl of the solution was injected at a rate of 2 μl/min through all cells. The monoclonal and the mouse sera were removed by flushing with 20 μl glycine-HCl, pH 1.5. Immobilised diphtheria toxin was removed from the sensor chip by 15 μl of 0.5 M DTT in 100 mM NaOH. By using the program BIAevaluation version 4.1., the raw data were evaluated in subsequent steps: the non-specific binding of serum was subtracted for the observed serum binding (signal of flow cell 4 was subtracted from 1, 2 and 3); the binding curves of serum were corrected for the amount of immobilised toxin, which varied slightly between the flow cells 1 to 3; the curves were simulated (using two state reaction model) to calculate maximal serum binding to the epitope-blocked diphtheria toxin (in flow cell 1 and 2), and maximal serum binding to non-blocked diphtheria toxin (in flow cell 3). The percentage of reduced binding was calculated by the ratio of both values.

Results

Selection of monoclonal antibodies

For the selection of monoclonal antibodies suitable for quality control of diphtheria toxoid, fifteen anti-diphtheria toxin monoclonal antibodies were available: seven antibodies that bind to the A-fragment of diphtheria toxin and eight antibodies that recognise the B-fragment. The antibodies can be divided in different groups that recognise non-overlapping epitopes. Therefore, SPR analyses were performed by capturing diphtheria toxin with a first antibody and measuring the binding of the second antibody. The binding of the second antibody was inhibited if the epitope overlapped with the first antibody (Table 1A and 1B). The measurements were also executed reversed order. However, a few inconsistencies were observed for monoclonals Dim 38 and Dim 40. The antibody Dim 38 did not bind to diphtheria toxin after capturing by Dim 42, whereas Dim 42 does after capturing by Dim 38. The epitope of Dim 40 overlapped with antibodies from groups IV and V (Table 2). In this chessboard inhibition SPR study, these monoclonals could be subdivided into five groups recognising a non-overlapping epitope (Table 2). From each group, one antibody was chosen for further studies: Dim 5, Dim 9, Dim 25, Dim 27, and Dim 33.

Toxin neutralising capacity

The selected antibodies were tested to demonstrate diphtheria toxin-neutralising activity. Monoclonals Dim 9 and Dim 25 showed the highest toxin-neutralising capacity (Table 3).

Table 1A. Inhibition study of monoclonal antibodies specific for A-fragment of diphtheria toxin.[a]

secondary primary	Dim 5	Dim 9	Dim 24	Dim 33	Dim 34	Dim 38	Dim 42
Dim 5	-[b]	+	-	+	N.D.	+	+
Dim 9	+[c]	-	N.D.	+	+	+	+
Dim 24	-	N.D.	-	N.D.	N.D.	N.D.	+
Dim 33	+	+	N.D.	-	-	-	N.D.
Dim 34	N.D[d]	+	N.D.	-	-	-	N.D.
Dim 38	+	+	N.D.	-	-	-	+
Dim 42	+	+	+	N.D.	N.D.	-	-

[a] For the inhibition study, diphtheria toxin was captured by a first monoclonal antibody. Subsequently, the binding of a second antibody to diphtheria toxin was measured.
[b] - = no binding of the second antibody (Response of second antibody / primary antibody £ 10%).
[c] + = substantial binding of the second antibody (Response of second antibody / primary antibody ≥ 10%).
[d] N.D. = not determined.

Table 1B. Inhibition study of monoclonal antibodies specific for B-fragment of diphtheria toxin.[a]

secondary primary	Dim 7	Dim 25	Dim 27	Dim 29	Dim 39	Dim 40	Dim 45	Dim 46
Dim 7	-	N.D.	-	-	N.D.	N.D.	N.D.	N.D.
Dim 25	N.D.	-	N.D.	N.D.	-	N.D.	N.D.	N.D.
Dim 27	-	N.D.	-	-	N.D.	N.D.	N.D.	N.D.
Dim 29	-	N.D.	-	-	+	-	-	+
Dim 39	N.D.	-	N.D.	+	-	-	+	-
Dim 40	N.D.	N.D.	N.D.	-	-	-	-	-
Dim 45	N.D.	+	N.D.	-	+	-	-	+
Dim 46	N.D.	N.D.	N.D.	+	-	-	+	-

[a] See legend to table 1A for explanation.

Table 2. Fifteen monoclonal antibodies divided in five groups.[a]

Group	Monoclonal antibodies	Reactive with fragment[c]
I	Dim 5[b] and Dim 24	A
II	Dim 9[b]	A
III	Dim 33[b], Dim 34, Dim 38, and Dim 42	A
IV	Dim 7, Dim 27[b], Dim 29, Dim 40 and Dim 45	B
V	Dim 25[b], Dim 39, Dim 40 and Dim 46	B

[a] Monoclonals within a group recognise an overlapping epitope; between groups the epitopes do not overlap (except for Dim 40, see test).
[b] Monoclonal antibodies selected for further immunochemical studies.
[c] Monoclonals bind to A-fragment or B-fragment of diphtheria toxin.

Table 3. The diphtheria toxin neutralising capacity of monoclonal antibodies.[a)]

Monoclonal antibody	Toxin neutralising concentration [b)] (μg/ml)
Dim 5	40.6
Dim 9	7.8
Dim 25	6.3
Dim 27	56.3
Dim 33	62.5
Dim 5, Dim 9, Dim 33	1.8 [c)]
Dim 25, Dim 27	2.7 [c)]
Dim 5, Dim 9, Dim 25, Dim 27, Dim 33	0.6 [c)]

[a)] Determined in toxin neutralisation test.
[b)] Antibody concentration for neutralising 5 ng/ml (0.0005 Lf/ml) diphtheria toxin.
[c)] The overall antibody concentration of the mixture is given (see section Materials en Methods for details).

They demonstrated toxin-neutralising activity at a 100-fold excess compared to diphtheria toxin. The immunoglobulins Dim 5, Dim 27 and 33 had little neutralising power, although these antibodies have high affinity for diphtheria toxin (17). Different mixtures prepared with these antibodies revealed a synergistic effect in toxin neutralisation. The neutralising power of the combined monoclonals was always better than that of the individual antibodies. In conclusion, neutralisation of diphtheria toxin is improved by the simultaneous binding of several antibodies against non-overlapping epitopes.

Anti-diphtheria toxin serum

For the selection of monoclonal antibodies that compete with antitoxin sera, first several immunochemical properties of sera were investigated. Three serum pools were obtained from eight mice immunised with vaccines A, B, or C. These diphtheria vaccines were prepared by different methods to induce a different immune response in mice (see section Materials & Methods). Indeed, the neutralising capacity varied for each of the pooled sera A, B, and C, as was analysed in an *in vitro* neutralisation test (Table 4). Serum A, which is from mice immunised with diphtheria toxin, was least potent. This phenomenon confirmed

Table 4. Immunochemical properties of mice against diphtheria toxin.

Serum	Toxin neutralising concentration [a)]	Avidity [M][b)] (μg/ml)	Ig[c)]	IgG[c)]	IgG1[c)]
A	50.0	1.1	3.6	3.7	4.0
B	12.5	1.0	3.6	3.5	4.1
C	3.5	1.4	3.7	3.5	4.4

[a)] Serum concentration for neutralising 5 ng/ml (0.0005 Lf/ml) diphtheria toxin.
[b)] NH_4SCN concentration resulting in 50% dissociation of antigen-antibody complex.
[c)] Data presented as log titres (see section Materials en Methods for details).

earlier findings (17). Serum C had the highest protecting activity. Moreover, the class and subclass -specific titres of the sera were determined (Table 3). The sera contained a relatively high concentration of IgG1-type antibodies, indicating the induction of a T helper 2 response. Titres of IgM, IgG2a, IgG2b and IgG3 could not be determined because of their low concentrations in the sera. Differences of 40% were found in the avidity of the sera for diphtheria toxin. Dissociation of antibodies occurred at an NH_4SCN concentration between 1.0 and 1.4 M (Table 4). The results showed that serum C, which has the highest neutralising power, also has the highest avidity. In conclusion, the sera pools revealed a different immunogenic profile.

Selection of monoclonal antibodies competing with antitoxin serum

To identify the immunodominant areas in diphtheria toxin, an epitope was first blocked by a monoclonal antibody. Subsequently, the binding of one of the sera was measured (Figure 2). Monoclonals that bind to an immunodominant epitope of diphtheria toxin should competitively inhibit the binding of serum antibodies. Binding of serum antibodies was reduced to 65% and 45% of the original values by the monoclonals Dim 5 and Dim 9, respectively (Figure 3). These results indicate that the immune response is largely directed to areas corresponding or close to the epitopes of Dim 5 and Dim 9. Both antibodies recognise an epitope on the A-fragment of the toxin. The other monoclonals Dim 25, Dim 27 and Dim 33 had a small contribution to prevent serum binding. For Dim 27 and Dim 33 this outcome was expected for sera B and C, because epitopes of both antibodies are almost completely destroyed in diphtheria toxoid as a result of formaldehyde treatment (17). Theoretically, serum A could have substantial amounts of antibodies directed to these

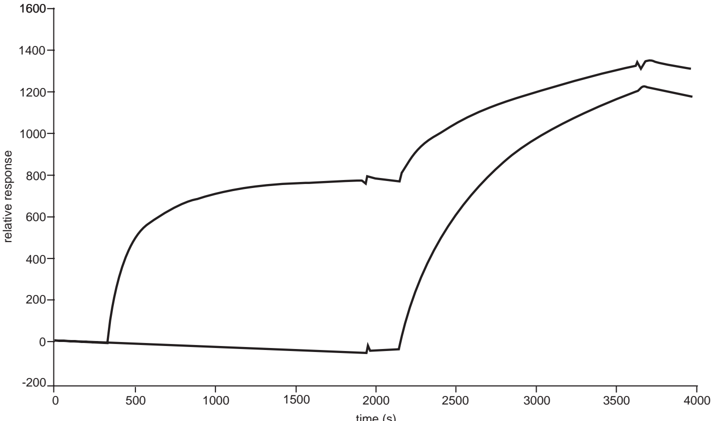


Figure 2. Simultaneous measurements of serum binding to diphtheria toxin in two different flow cells 2 and 3. Monoclonal Dim 9 was injected for 25 min (350 – 1850 s) through flow cell 1 and the binding was measured. Subsequently, mouse antitoxin serum was injected for 25 min (2200 – 3700 s) through flow cells 2 and 3 which are connected in series. The figure shows a reduced serum binding in flow cell compared to flow cell, as a result of epitope blocking by Dim 9.

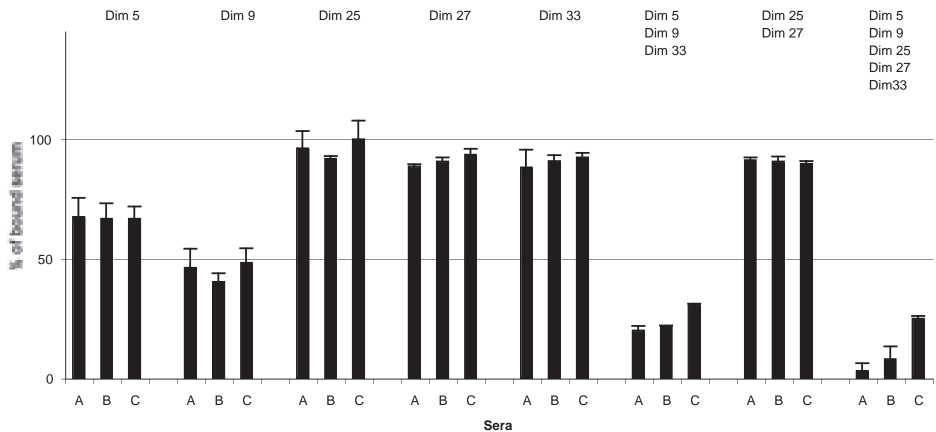


Figure 3. Reduced serum binding to diphtheria toxin. Five monoclonal antibodies in different combinations were used to block epitopes on diphtheria toxin. Depending on the used antibody, substantial reduction in toxin binding was observed for serum A, B and C (Mean ± S.D.; n = 4).

epitopes, because this serum was obtained from mice immunised with diphtheria toxin. This was not the case, indicating that these epitopes are not very immunogenic. The combination of the five antibodies could almost completely inhibited the binding of sera. Thus, all immunogenic sites in diphtheria toxin could be covered by these five monoclonal antibodies (Figure 3).

In conclusion, the antibodies Dim 5 and Dim 9, which bind to immunodominant epitopes of diphtheria toxin, are important candidates for the potency determination of diphtheria toxoid vaccines. On the other hand, Dim 27 en Dim 33 were highly suitable to monitor the inactivation process of diphtheria toxin, as was earlier described [16].

Discussion

Different immunochemical properties of monoclonal antibodies were studied to select potential candidates for the quality control of diphtheria toxoid vaccines. At first, SPR-analyses identified five groups of antibodies, which bind to non-overlapping epitopes on diphtheria toxin (Table 1). From each group, an antibody was chosen for further studies: Dim 5, Dim 9, Dim 25, Dim 27 and Dim 33. Toxin neutralising capacity was determined and the monoclonals were used to identify immunodominant epitopes on diphtheria toxin. Monoclonals Dim 9 and Dim 25 have relatively high neutralising activity (Table 4). Furthermore, Dim 5 and Dim 9 inhibited to a considerable extent the binding of mouse antitoxin serum to diphtheria toxin (Figure 3).

Our results indicate that diphtheria toxin has at least five unique epitopes. However as reported in the past, investigations with monoclonals showed that at least ten distinct

epitopes are located on the toxin molecule (15). Apparently, the limited number of monoclonals did not contain all specificities. On the other hand, biosensor analyses showed that these antibodies successfully competed with mouse antitoxin serum inhibiting almost completely binding of anti-diphtheria toxin sera (see Figure 3). Hence, we assume that most immunogenic sites in diphtheria toxin can be monitored by the five monoclonal antibodies selected in this study.

A number of recent studies have described serum analysis by using surface plasmon resonance (21-27). This technique has been exploited for diagnosis of diseases, analysis of antibody titres after vaccination, and examination of unintended immunogenicity induced against therapeutic proteins. Biosensor analysis is less often used for the detection of immunodominant epitopes. In this study, the technique is utilised to evaluate on epitope level the binding of antiserum to diphtheria toxin. The experiments revealed that a large proportion of the mouse anti-diphtheria toxin antibodies in the serum was directed against the A-fragment, because monoclonals Dim 5 and Dim 9 inhibited to a considerable extent the binding. However, the exact positions of the epitopes for these antibodies are still unknown. The other monoclonals, Dim 25, Dim 27 and Dim 33, competed with antitoxin serum to a much lesser degree, although these antibodies demonstrated other important properties. Dim 25 has high toxin-neutralising activity, whereas Dim 27 and Dim 33 recognise formaldehyde sensitive epitopes.

Rolf et al. reported that high concentrations of toxin-neutralising immunoglobulins are necessary for protection; 100-fold molar excess of monoclonal antibodies compared to diphtheria toxin (14). This observation agreed with our results. Antibodies Dim 9 and Dim 25 demonstrated toxin neutralising activity at a 100-fold excess. For the other monoclonals even much higher concentrations were necessary for toxin neutralisation. A synergistic effect in neutralising activity was shown when combinations of monoclonal antibodies were used. Probably, this can be explained by preventing several biological actions of the toxin, such as avoiding the receptor binding, blocking the entry into the cytosol, or inhibiting the catalytic activity.

Beside monoclonals Dim 27 en Dim 33 for monitoring the detoxification process [16], monoclonal antibodies Dim 5, Dim 9 and Dim 25 are candidates for a surrogate potency test of diphtheria toxoids. To that end, we will qualify the procedure and subsequently we will develop an easy to use ELISA by using these five monoclonal antibodies. In this assay(s), the comparability of a new toxoid batch with a reference toxoid with known in vivo potency can be determined. After extensive validation the method may be usable for release of diphtheria toxoid batches when combined with a series of physicochemical and biochemical tests (17).

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Chapter 9

Summary and perspectives

Summary

The primary concern of the vaccine industry is to produce vaccines with consistent high quality. To that end, control tests are performed in all stages of manufacturing from starting materials through final vaccine lots. Many animal-based tests are included to determine the potency and safety of human vaccines. In Europe, approximately 1.5 million laboratory animals are being used every year for routine quality control of vaccines (1). Manufacturers, regulatory authorities and ethical commissions wish to minimise animal use, because of a number of serious drawbacks, including cost, imprecision, and ethical concerns. Currently, the 3R concept: 'replacement, reduction and refinement of animal tests' is the guiding principle for existing regulations on animal experimentation and stimulates the development of new characterisation methods (2-4).

This thesis presents the results of a study to structurally characterise diphtheria toxoid with physicochemical and immunochemical techniques. The modifications that result from the formaldehyde treatment were investigated for a better understanding of the detoxification process. This knowledge may help to develop new *in vitro* tests, as an alternative for the current *in vivo* potency test.

Chapter 2 provides a general overview of the opportunities and problems in the reduction of animal use for human vaccine quality control. Progress in the reduction of animal tests with regard to *in vivo* potency and safety testing is discussed. Furthermore, a fingerprint approach is proposed as a strategy to demonstrate the quality of vaccines. A combination of several physicochemical and immunochemical tests should make it possible to determine the comparability between a routine vaccine batch and a reference preparation with a well-known potency. When both products are indistinguishable, the potency of the new batch should be comparable. Many analytical techniques are described that are suitable to investigate properties of antigens, i.e. the identity, size, secondary and tertiary structure, and purity.

Chapter 3 describes the possibility of monitoring the quality in diphtheria vaccine production with several physicochemical and immunochemical tests described in chapter 2, as an alternative to established *in vivo* tests. The *in vitro* assays were performed with experimental toxoids obtained from one toxin batch. A clear correlation was found between these tests and the standard potency and safety tests. All these techniques revealed structural differences between diphtheria toxin and toxoid. This makes them suitable to monitor the most critical production step: the inactivation of the diphtheria toxin by formaldehyde. In the study a number of criteria were proposed that diphtheria toxoid has to meet, i.e. an apparent shift of the B-fragment in SDS-PAGE, a reduction of the number of primary amine groups, an increased resistance to denaturation, an increased circular dichroism signal in the near-UV region, and a reduced binding to selected monoclonal antibodies. This indicates

that it may be possible that a selected set of *in vitro* analyses can replace the classical *in vivo* tests to evaluate the quality of diphtheria toxoid vaccines, provided that the validity of these tests is demonstrated in extensive validation studies and regulatory acceptance is obtained.

Chapter 4 presents the quality control of thirteen regular vaccine batches of different manufacturers and three experimental toxoids, prepared by adapted inactivation procedures. Some of these products were unique with respect to their age: production dates dated back to 1967. The results justify further evaluation of the test battery because of their high precision and easy performance as compared to conventional procedures with test animals.

C**hapter 5** shows the chemical modifications of reactive amino acid residues occuring during the formaldehyde treatment. Synthetic model peptides were analysed by liquid chromatography-mass spectrometry (LC-MS) after treatment with formaldehyde under different conditions. The study revealed that formaldehyde forms labile methylol adducts and Schiff-bases with N-terminal amino acid residues and the side-chains of arginine, cysteine, histidine, lysine and trypthophan residues. Importantly, formaldehyde treatment causes stable cross-links between primary amino groups and several amino acid residues including arginine, asparagine, glutamine, histidine, tyrosine and tryptophan. The use of model peptides enabled us to determine the reactivity of each particular cross-link reaction as a function of the reaction conditions and to identify newly discovered reaction products after incubation with formaldehyde.

Chapter 6 concentrates on the study to map the formaldehyde-induced modifications in a model protein, insulin. The purpose of this study was to investigate if the results obtained with the peptides (chapter 5) can be applied to identify the chemical modifications in a relatively small protein before studying the more complex protein diphtheria toxoid (chapter 7). In theory, insulin has sixteen potential reactive sites, whereas diphtheria toxoid has hundred and forty. In this study, formaldehyde-treated insulin was digested with proteinase Glu-C and fragments were analysed by LC/MS. The results confirmed the study with model peptides (chapter 5), but also indicated that the protein conformation affects the accessibility and reactivity of susceptible residues in insulin.

Chapter 7 focuses on the cross-links in diphtheria toxin after formaldehyde and glycine treatment. A detailed analysis of formaldehyde-induced modifications was done for the functional sites of diphtheria toxin, i.e. the NAD^+-binding cavity and the receptor-binding site. It was shown that intramolecular cross-links and formaldehyde-glycine attachments were formed in both the NAD^+-binding cavity and the receptor-binding site. The modifications in these functional sites probably result in the detoxification of diphtheria toxin.

Chapter 8 presents a study in which a basis is provided for an antigenicity assay as a possible substitute for *in vivo* immunogenicity measurements. Therefore, monoclonal antibodies were selected that bind to immunodominant epitopes. Five monoclonal antibodies were selected after it was demonstrated that they recognise non-overlapping epitopes. In a biosensor assay, the combination of antibodies, Dim 5, Dim 9, Dim 25, Dim

27 and Dim 33, could (almost) completely inhibit the binding of mouse antiserum to diphtheria toxin. Especially monoclonal antibodies Dim 5 and Dim 9 demonstrated substantial inhibition of antiserum binding, indicating that they recognise an immunodominant epitope. Furthermore, monoclonals Dim 9 and Dim 25 showed the highest toxin neutralising activity. These monoclonal antibodies are recommended to be used in immunochemical assays, as a possible substitute for the *in vivo* potency test of diphtheria vaccines.

Perspectives

The potency of diphtheria toxoid vaccines is determined by the antigen structure and the vaccine composition. During the detoxification process, the structure of diphtheria toxin changes drastically by the formaldehyde-induced modifications. Hence, the inactivation step affects the potency of the resulting diphtheria toxoid. The potency of a freshly prepared vaccine batch is routinely tested in an intradermal challenge test with guinea pigs or a serological assay with mice. Numerous disadvantages of *in vivo* tests justify the quest for alternative methods. Especially, physicochemical and immunochemical techniques are the ultimate tools to study the changes in the antigen structure. For that reason, they are potential candidates to act as alternatives for the potency tests.
In general, no single analytical technique can completely characterise diphtheria toxoid. The techniques are restricted to elucidate one or a few properties of the toxoid, e.g. a typical chemical modification, a change in the secondary or tertiary structure, or the damage to an epitope. The use of several non-related techniques is necessary to characterise the structure of diphtheria toxoid, resulting in a fingerprint of the antigen. The fingerprint can be compared with that of a well-known reference preparation to demonstrate comparability. But, a crucial question related with the aim of this thesis remains: will it be possible to use these *in vitro* tests as a surrogate for the potency tests, i.e. does the fingerprint provide us with quantitative data? To answer this question, one has to realise that diphtheria toxoid is released when the potency according to the *in vivo* test is at least 2 IU/Lf (and all other release criteria are met). Here we are confronted with the complication that it is not possible to determine the potency in specified units (IU/Lf) with the *in vitro* alternatives. However, this thesis indicates that –in principle– the fingerprint approach can guarantee that the minimally required potency is present. Therefore, the concentration and structural integrity of diphtheria toxoid have to be assessed in the final batch (bulk).
Still, this pass/fail assessment has some hurdles to take before it can be used on a routine basis. The first problem is that the precise and sensitive *in vitro* tests have to correlate with the imprecise and insensitive *in vivo* tests (see chapter 4). It is likely that the level of correlation between *in vitro* and *in vivo* tests cannot be determined unless *in vivo* experiments are repeated very often or the number of animals per experiment is increased

substantially. Only then small potency differences between batches can be detected *in vivo*. The second problem here is that diphtheria toxoid is a very robust and stable antigen (see chapter 4). For the qualification and validation of *in vitro* tests it is necessary to have access to 'out of specification' products and/or borderline toxoids. These are difficult to produce. From a manufacturers point of view this is obviously advantageous, but for implementation of new quality control methods this is a serious drawback. With the increased knowledge about the effect of different inactivation procedures (e.g. acetylation, methylation; chapter 4) it may be possible to prepare borderline products. In any case, more intensive animal studies may be necessary in order to qualify the panel of *in vitro* tests. The results in chapters 3 and 4 indicate that this has a high chance of success.

How would a future test panel for diphtheria toxoid look like? As mentioned in chapters 2 and 3 several physicochemical and immunochemical analyses are necessary. Because of their simplicity, the use of SDS-PAGE, primary amine group determination and ELISA is recommended. In these tests, the freshly prepared diphtheria toxoid should be compared with the parent diphtheria toxin and a reference toxoid with a well-known potency. These techniques can assess the extent of the action of formaldehyde by the width of the toxoid band, the number of amino groups left and the disappearance of epitopes in diphtheria toxin. Furthermore, ELISA with specified monoclonal antibodies (chapter 8) can be used to determine that immunodominant epitopes are intact after the detoxification with formaldehyde. Other techniques, such as fluorescence, circular dichroism, SPR-biosensor analysis and combined liquid chromatography, electrospray ionisation mass spectrometry, have to be considered for quality control, but are still not routinely used in quality control protocols.

Two immunodominant epitopes in diphtheria toxoid were identified by anti-diphtheria monoclonal antibodies, Dim 5 and Dim 9 (chapter 8). The exact locations of these epitopes are still unknown, but could be determined, for example, by alanine scanning mutagenesis (5). Especially, the formaldehyde-induced modifications at the immunodominant sites are very interesting. They could be elucidated by LC-MS analyses after digestion of the toxoid. Further investigations are necessary, but perhaps the analysis of immunodominant epitopes with SPR-biosensor analysis and LC-MS are sufficient to predict the immunogenicity of diphtheria toxoid. To simplify the search for modified residues by LC-MS analyses, equal amounts of a freshly prepared toxoid and a reference preparation, treated with deuterium-labelled formaldehyde, should be mixed prior to the digestion. The peptides with formaldehyde modifications appear as mass spectral doublets during the LC-MS analysis. Furthermore, if the modifications in the new toxoid strongly vary from the reference preparation, no doublets will be observed.

After development of the *in vitro* tests, two important hurdles have to be taken before a new test method can be implemented as alternative for animal tests: extensive validation must demonstrate the robustness of each test, and finally regulatory acceptance is necessary. Thus, whether these tests are accepted for routine controls depends on the outcome of the

validation study. The validation of new test methods is a major task in the process from test development to implementation. For an in-process or a pre-screening test, validation is relatively simple and can be confined to an in-house study and a publication in a peer reviewed journal. However, validation of a new method that is considered for regulatory acceptance (e.g., a final release test) is tedious and time-consuming. Before such a new test can be applied, it has been evaluated in four main stages: pre-validation (an informal inter-laboratory study), validation (a formal inter-laboratory study), independent assessment, and regulatory acceptance. The period between development and acceptance of new tests may take up to 15 years (6, 7).

The *in vitro* tests have two possible applications: as an in-process quality control or as release test for the final lot. The quality of diphtheria toxoid is tested in several stages of the production process. Since several final lots are subsequently produced from one diphtheria-toxoid batch, the potency and safety of the batch are repeatedly tested in animals. Physicochemical and immunochemical tests are very suitable to demonstrate that a toxoid batch, which is tested immediately after production, is comparable to this batch after storage for, e.g., one year. If this approach is accepted by regulatory authorities this may lead to substantial reduction of animal use in release testing of toxoid vaccines. Thus, based on *in vitro* analytical tests a toxoid batch (bulk) may be re-released for subsequent preparation of final products without additional *in vivo* testing.

The release of the final vaccine lot is based on the results of the potency and safety tests. In the final lot, diphtheria toxoid is adsorbed to aluminium phosphate or aluminium hydroxide. In addition, the toxoid is often mixed with other antigens. This influences the potency of the diphtheria component. It is very difficult, if not impossible, to characterise diphtheria toxoids by physicochemical and immunochemical tests in such formulations. The quality of toxoid has to be determined by the *in vitro* tests prior to adsorption and mixing. Otherwise, the toxoid has to be desorbed from aluminium phosphate or aluminium hydroxide with the assurance of maintained structural integrity. A way to overcome this problem is to validate the reproducibility of the preparation of final lots. This means that (i) the quality of the adjuvant and excipients has to be constant and (ii) the process steps during mixing and filling are robust and well validated.

The development of *in vitro* functional tests has to be considered for the quality control of the final vaccine lot. These functional tests are based on the use of cells of the immune system that are kept in culture. A collection of various cell types is necessary to mimic the complex immune system. The final vaccine lot has to induce an immune response in *in vitro* cell cultures. The development of such techniques will be a challenge in near future.

The methods described in this thesis are probably also suitable for the characterisation of other formaldehyde antigens like tetanus toxoid and the inactivated polio vaccine. Especially for the development of a new inactivated polio vaccine, based on the safer attenuated Sabin strains (8), this study can be helpful to define a suitable inactivation matrix. Formaldehyde is not only used as an inactivating agent but also as stabiliser, for

instance for genetically detoxified pertussis toxin (9). Pertussis toxin was treated with low formaldehyde concentrations. The treatment resulted in an increased immunogenicity.
In conclusion, it may be possible in the long run to diminish or even abolish *in vivo* release tests for diphtheria vaccines when, beside the structural characterisation of diphtheria toxoid, the composition of the vaccine can be defined and quantified. Currently, the release of a number of vaccines, e.g. hepatitis A and hepatitis B vaccines, is based on *in vitro* assays. These vaccines are well-defined biologicals, but this thesis demonstrates that the release based on *in vitro* tests may also be possible for classical antigens like diphtheria toxoid.

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Samenvatting

Samenvatting

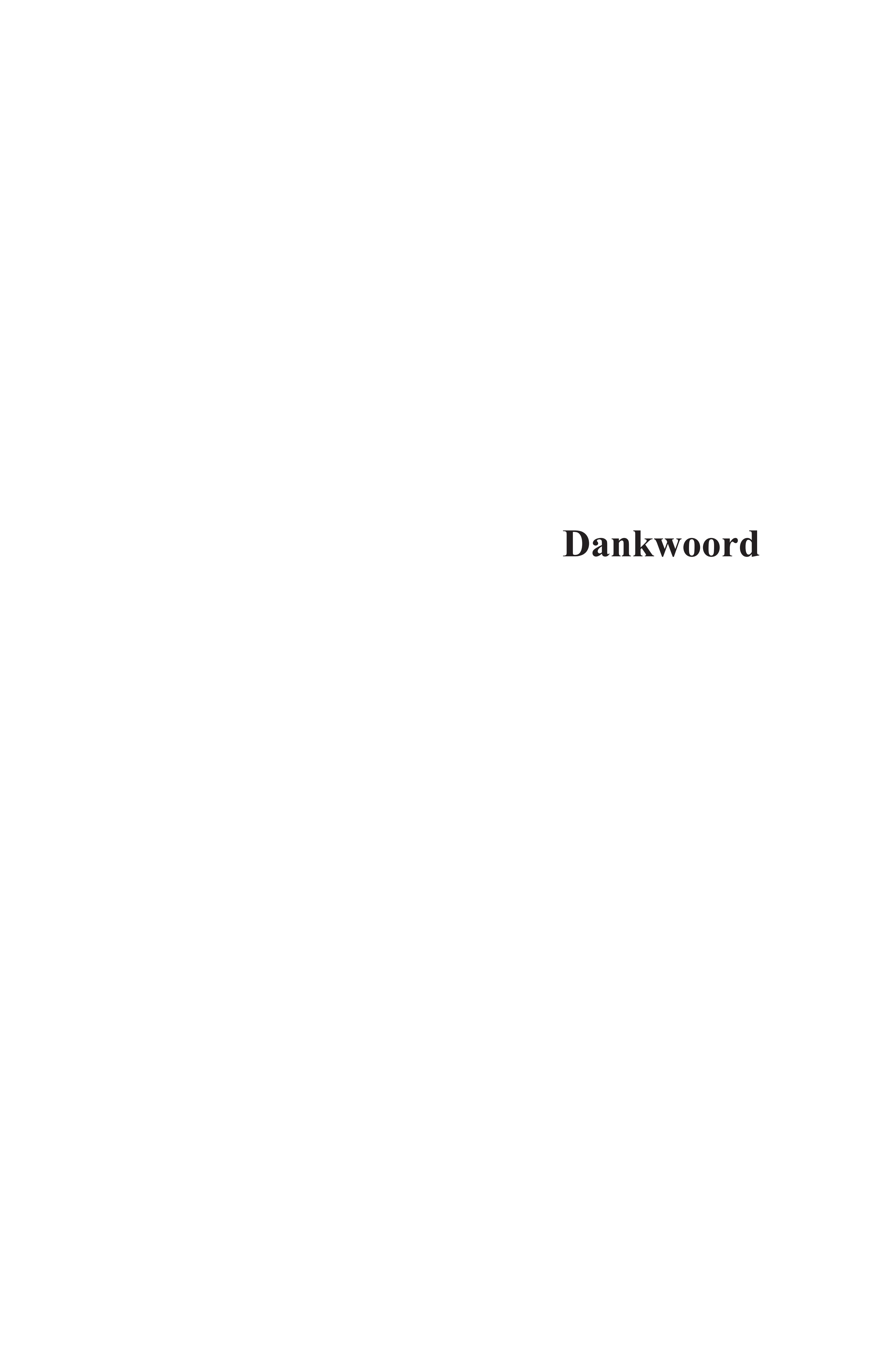
Het grootste belang van de vaccinindustrie is het vervaardigen van vaccins van een stelselmatig hoge kwaliteit. Daarom worden in verschillende stadia van de vaccinproductie kwaliteitscontroles uitgevoerd. Dit begint met het testen van grondstoffen, vervolgens controletesten tijdens het productieproces en eindigt met analyses op het eindproduct. Op grond van alle testuitslagen wordt een partij van het vaccin uiteindelijk vrijgegeven. Voor een aantal testen worden proefdieren gebruikt ter vaststelling van de veiligheid en de werkzaamheid van het vaccin. Veel mensen en instanties, inclusief fabrikanten en overheidsinstanties zien graag een vermindering in het gebruik van proefdieren vanwege de hoge kosten, de onnauwkeurigheid van de testen en de ethische bezwaren die voortvloeien uit het gebruik van proefdieren. De ontwikkeling van nieuwe analysetechnieken voor de kwaliteitscontrole heeft in een aantal gevallen geleid tot een vermindering en verfijning in het proefdiergebruik. Desondanks is het aantal benodigde proefdieren voor de kwaliteitscontrole van vaccins omvangrijk. Jaarlijks worden hiervoor in Europa ongeveer 1,5 miljoen proefdieren gebruikt. Vooral de werkzaamheidstesten voor klassieke vaccins vragen grote aantallen proefdieren. Zo wordt bijvoorbeeld iedere productiepartij van difterievaccin getest op tachtig tot honderd muizen of cavia's ter vaststelling van de werkzaamheid voordat die partij wordt vrijgegeven.
Voor het hier beschreven onderzoek is difterietoxoïd gebruikt omdat het een veelgebruikt klassiek vaccin is. De ziekte difterie wordt veroorzaakt door een bacterie (*Corynebacterium diphtheriae*) die zich kan handhaven in de keelholte en op de huid van de mens. De bacterie produceert een gifstof, difterietoxine geheten, die weefsels beschadigt van luchtwegen en hart en het zenuwstelsel aantast. De ziekteverschijnselen die optreden bij difterie kunnen keelpijn, opgezwollen hals (burgemeestersnek) en koorts zijn. Het meest kenmerkend van difterie is de vorming van een grijs membraan in de keelholte. In een vergevorderd stadium van de ziekte treden ook verlammingsverschijnselen en ademnood op. Gelukkig is na de invoering van difterievaccinatie in 1953 het aantal ziektegevallen in Nederland sterk gedaald van gemiddeld drieduizend naar één geval per jaar.
De bereiding van het difterievaccin begint met het grootschalig kweken van difteriebacteriën in een fermentor met vloeibaar groeimedium. Tijdens de groei wordt door de bacteriën difterietoxine aan het medium afgegeven. Na het stopzetten van de kweek wordt een aantal handelingen uitgevoerd met het difterietoxine. Als eerste wordt het medium met het difterietoxine gescheiden van de bacteriën. Vervolgens wordt het toxine in het medium geconcentreerd. Daarna volgt een belangrijke stap, namelijk de inactivering van het toxine met formaldehyde. Het gevormde product wordt difterietoxoïd genoemd en is de werkzame stof in het vaccin, die het lichaam aanzet tot het produceren van beschermende antistoffen tegen difterie. Het vaccin, dat bestaat uit gezuiverd difterietoxoïd en een aantal toegevoegde hulpstoffen in een geschikte verpakking, wordt vaak gecombineerd met andere vaccins, zoals kinkhoest-, polio- en tetanusvaccin.

Het in dit proefschrift beschreven onderzoek richtte zich hoofdzakelijk op het vaststellen van de werkzaamheid van het difterievaccin met behulp van een aantal analytisch-chemische technieken als een mogelijk alternatief voor de traditionele dierproeven. Van deze technieken is onderzocht of ze in staat zijn om verschillen aan te tonen in de structuur van het difterietoxoïd. Zo ja, dan kunnen ze in principe gebruikt worden om de werkzaamheid van het vaccin vast te stellen, omdat deze met name bepaald wordt door de structuur van het difterietoxoïd.

De werkzaamheid van klassieke vaccins, zoals difterievaccin, wordt met gebruikmaking van proefdieren vastgesteld. Bij andere vaccins, zoals o.a. voor het hepatitis B-vaccin en het geïnactiveerde griepvaccin, mogen analytische technieken worden gebruikt om ze te kunnen vrijgeven voor gebruik. Hiervan worden in de literatuur talrijke voorbeelden gegeven (samengevat in **Hoofdstuk 2**). Tijdens dit onderzoek is gezocht naar analytische technieken die geschikt zijn om de kwaliteit van het difterievaccin te bepalen. Er zijn vijf analytisch-chemische testen geselecteerd: gel-electroforese, vrije-aminogroepbepaling, denaturatietest, circulair dichroïsme en biosensoranalyse. Hiermee kunnen verschillende eigenschappen worden vastgesteld van difterietoxoïd, zoals de grootte van de toxoïdmoleculen, de mate van inwerking van formaldehyde, de secundaire en tertiaire structuur, en de veranderde bindingsplaatsen voor antistoffen. Met behulp van een aantal experimentele difterievaccins zijn criteria vastgelegd, waaraan vaccins moeten voldoen (**Hoofdstuk 3**). Vervolgens zijn deze waarden getoetst met toxoïden van verschillende producenten, en met verouderde en afwijkende toxoïden (**Hoofdstuk 4**). Uit het onderzoek blijkt dat met de bovengenoemde technieken structurele verschillen in het toxoïd kunnen worden aangetoond.
Het inactiveren van difterietoxoïd is een beslissende stap in de productie die in grote mate bepalend is voor de kwaliteit van het uiteindelijke vaccin. Formaldehyde tast namelijk de structuur aan van difterietoxine doordat het chemisch met bepaalde aminozuurresiduen van het eiwit reageert. De chemische reacties die op kunnen treden tussen formaldehyde en eiwitten waren slechts ten dele bekend. Met behulp van massaspectrometrische analyse van een serie synthetische peptiden met tevoren geselecteerde aminozuurvolgorden zijn de voornaamste reactieproducten geïdentificeerd die tijdens de formaldehydebehandeling kunnen ontstaan (**Hoofdstuk 5**). Deze informatie is vervolgens gebruikt om de chemische veranderingen op te sporen in twee met formaldehyde behandelde eiwitten, insuline en difterietoxine. In eerste instantie is voor een modeleiwit, insuline, gekozen omdat het relatief klein is; er kunnen ‘slechts’ zestien aminozuren met formaldehyde reageren (**Hoofdstuk 6**). Met behulp van een massaspectrometer zijn de veranderingen in insuline geïdentificeerd. Bij difterietoxine, dat ongeveer tien keer zo groot is als insuline, is vooral gekeken naar de chemische veranderingen op twee posities in het molecuul die betrokken zijn bij de werking van het eiwit. Het bleek dat tijdens het inactiveren de twee functionele

plaatsen in het difterietoxine worden aangetast door formaldehyde (**Hoofdstuk 7**). De plaatsen op het difterietoxoïdmolecuul die herkend kunnen worden door antistoffen worden epitopen genoemd. Zulke epitopen bevinden zich aan de oppervlakte van het eiwit. Deze epitopen dienen zeer waarschijnlijk intact te blijven tijdens de bereiding van het difterietoxoïd. Er zijn twee epitopen gevonden waar de meeste antistoffen uit het bloed (serum) van met difterietoxoïd geïmmuniseerde muizen aan binden (**Hoofdstuk 8**). Met gebruikmaking van een aantal speciaal geselecteerde antistoffen kan de aanwezigheid van deze epitopen in routineproducten door middel van immunochemische analyses worden geverifieerd.

Samenvattend heeft het onderzoek geleid tot de beschikbaarheid van een aantal analytische testen om de kwaliteit van difterietoxoïden te bepalen en tot een diepgaande kennis van het inactiveringsproces van difterietoxine. Een groot aantal structurele eigenschappen van het difterietoxoïd is met deze studie opgehelderd. De in dit proefschrift beschreven inzichten zullen in de toekomst hopelijk bijdragen aan een verbeterde kwaliteitscontrole van het difterievaccin, waarbij minder proefdieren nodig zullen zijn.
Daarnaast vergemakkelijken de resultaten van dit onderzoek de ontwikkeling van soortgelijke analyses voor andere met formaldehyde geïnactiveerde vaccins, zoals tetanustoxoïd en geïnactiveerd poliovaccin.



Dankwoord

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Sytse, ik ben blij dat je voor mij vele figuren uit dit proefschrift hebt gemaakt.
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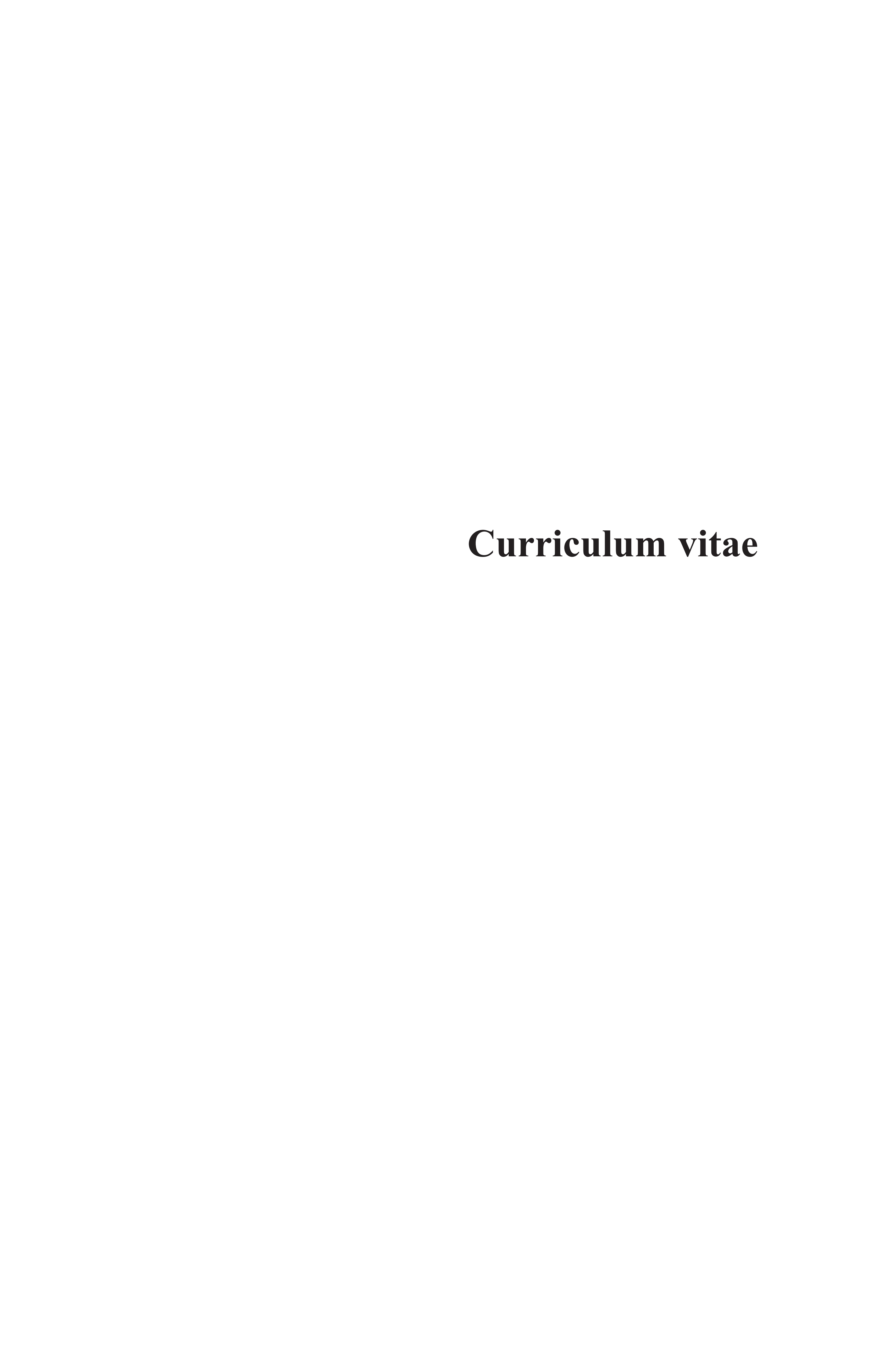
Familie en vrienden, jullie hebben mij in de afgelopen periode erg gesteund. Femke, Henny, Gerrit en …, ik hoop er nu voor jullie te zijn.



Curriculum vitae

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Bernard Metz was born in November 18th 1972 in Dokkum, The Netherlands. In 1993 he finished high school (VWO) at the 'Lienward College' in Leeuwarden and started with the study Chemistry of the Faculty of Mathematics and Natural Sciences, University of Groningen. During his study he participated in two research programs entitled: 'Signal transduction in oligodendrocytes' at the Department of Physiological Chemistry, University of Groningen, and 'Random mutagenesis of the human serotonin receptor in *Dictyostelium discoideum*' at the Department of Biochemistry, University of Groningen. He graduated in 1998. From 1999 until 2000, he was involved as research associate in the project entitled 'Random mutagenesis of gonadotrophins in *Dictyostelium discoideum*' (a collaboration between the Department of Biochemistry, University of Groningen and Organon, Oss). In the period between 2000 and 2004, he was working as graduate student under the supervision of Dr. W. Jiskoot, Dr. G.F.A. Kersten, Prof. Dr. W.E Hennink and Prof. Dr. D.J.A. Crommelin on his thesis Structural Characterisation of diphtheria toxoid. This project was a collaboration between the Department of Pharmaceutics of the Faculty of Pharmaceutical Sciences of the Utrecht University and the Unit Reseach and Development of the Netherlands Vaccine Institute in Bilthoven. Since 2004, he is working at the Netherlands Vaccine Institute.