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Characterization of production processes for tetanus and diphtheria anatoxins

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ABSTRACT

Tetanus and diphtheria are diseases that still cause significant morbidity and mortality. *Clostridium tetani* produces the tetanus toxin, a 150-kDa protein. The diphtheria toxin is synthesized by *Corynebacterium diphtheriae* as a protein of 58 kDa. The objective of this study was to carry out a chemical characterization of the tetanus and diphtheria toxin forms in the several production process stages, and thus to establish an affordable alternative *in vitro* quality control to aggregate to the classical tests. The 150 kDa band of the tetanus toxin and approximately 58 kDa band of the diphtheria toxin were observed by electrophoresis similar as that described in the literature. The same band of 58 KDa was detected in Western blotting reactions. The results obtained for diphtheria toxin showed very similar protein profiles between distinct lots. For the tetanus toxin, the profiles of the initial stage showed some variability, but the ones of the following stages were similar. The similarity of the electrophoresis results indicated reproduction and consistency of the production processes in Butantan Institute and correlated with the yield and antigenic purity classical data. The establishment of alternative *in vitro* quality control tests can significantly contribute to achieve the consistency approach supported by WHO.

1. Introduction

Tetanus and diphtheria are important illnesses that still cause significant morbidity and mortality in people worldwide that have not been vaccinated or have incomplete immunization.

Clostridium tetani is an anaerobic gram positive bacillus responsible for the production of a potent neurotoxin called tetanus toxin (TeNT), which is released into the medium after autolysis and leads to tetanus disease [1–3]. Mature TeNT has a molecular mass of 150 kDa and is composed of two chains, one of 100 kDa (heavy chain) and the other of 50 kDa (light chain), linked by a disulfide bond [4–6]. TeNT mechanism of action includes cell binding, internalization, trafficking, translocation to neuronal cytosol and proteolytic cleavage of a substrate (VAMP – vesicle associated membrane protein) from the SNARE (soluble NSF (N-ethyl-maleimide-sensitive fusion protein) attachment protein receptor) complex [7–9].

Corynebacterium diphtheriae is an aerobic bacterium that produces an exotoxin, diphtheria toxin, which is the causative agent of diphtheria [10]. It is synthesized as a precursor form of 61 kDa. The secreted protein has a molecular mass of 58 kDa and is composed of two distinct fragments: A of 21 kDa and B of 37 kDa, linked by disulfide bonds [11–13]. There are three main steps of diphtheria toxin mechanism of action: binding to receptors, translocation of the catalytic domain to the cytosol and inhibition of protein synthesis involving EF2 (elongation factor 2) [13–15].

In 1924, Ramon described that toxins, such as diphtheria and tetanus, could be inactivated by formaldehyde treatment resulting in nontoxic forms (toxoids or anatoxins). This finding permitted the beginning of the production of the first vaccines by chemical treatment [16]. Butantan Institute started to produce anti-tetanus serum in 1911 and the development of tetanus and diphtheria vaccines in 1924 and 1930, respectively [17].

Toxoid vaccine production processes, such as for tetanus and diphtheria, involve the cultivation of the microorganisms, the extraction, concentration and inactivation of the toxins, and finally, the purification of the toxoids. Each of these steps should be monitored to control the quality of the products and ensure a high level of efficacy and safety [18,19]. The establishment of *in vitro*

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quality control protocols for vaccine production has been an important research field worldwide [21–23], using analytical chemistry (such as chromatography and SDS-PAGE) or immunochemical (such as ELISA, TOBI (toxin binding inhibition) and Western blotting) methods with the aim to reduce animal use for *in vivo* quality control assays. Analytical chemistry characterization (as SDS-PAGE and Western blotting) of tetanus and diphtheria anatoxins was described only to two stages, the toxin and the final product, until this moment [23,24]. In this study, we describe for the first time the chemical characterization of the main steps of the whole tetanus and diphtheria anatoxins production processes, using SDS-PAGE and Western blotting to establish affordable *in vitro* quality control assays to aggregate to the classical tests and provide a better characterized product profile.

2. Materials and methods

2.1. Toxin and anatoxin

Tetanus and diphtheria toxins or anatoxins were produced at the Butantan Institute (São Paulo, SP, Brazil) as recommended by WHO [18,25,26]. *C. tetani* was cultivated in medium described by Mueller and Miller [27] and modified by Latham and coworkers [28] at 36 °C ± 1 °C. *C. diphtheriae* was grown in medium described by Stainer et al. [29] at 36 °C ± 1 °C. The production processes of these anatoxins were briefly described in Subsection 2.2.

2.2. Tetanus and diphtheria anatoxins production procedures

The processes started with the cultivation (C) of *C. tetani* or *C. diphtheriae* under fermentation conditions, and the supernatants of the cultures obtained by a tangential filtration step and concentration of the tetanus or diphtheria toxin (30 kDa cut-off membranes). The concentrated (CT) tetanus or diphtheria toxin was then inactivated by the addition of chemical reagents (formaldehyde) and heat (37 °C). Following the approval of the Internal Quality Control Service, the bulk (B) tetanus and diphtheria anatoxins were submitted to a purification procedure. Tetanus bulk anatoxin (M), a result of the mixture of 3 lots of (B), was concentrated and diafiltered (CD) through 50 kDa cut-off membranes and then purified by gel filtration chromatography. The resulting product (concentrated and purified (P) tetanus anatoxin) was filter-sterilized and sampled by the Internal Quality Control Service. The lots of concentrated bulk anatoxin that are approved by Internal Quality Control Service are called tetanus anatoxin final bulk (FB). Bulk diphtheria anatoxin (M), a result of the mixture of 2 lots of (B), was also concentrated and diafiltered (CD), but using 30-kDa cut-off membranes. The purification was carried out by ammonium sulfate precipitations and a new step of diafiltration and concentration. Concentrated and purified (P) diphtheria anatoxin follows the same steps of tetanus anatoxin, and the final product is called diphtheria anatoxin final bulk (FB).

A summary of the production processes is presented in Fig. 1.

2.3. Yield and antigenic purity

Yield (antigen concentration) data was obtained from determination of concentration in Lf (Limit of flocculation or *limes flocculationis*)/mL by flocculation test. Antigenic purity data was determined by the ratio of the antigen concentration and the concentration of protein (nondialysable) nitrogen as recommended by WHO [18,20]. The graphic representations and the column statistics in Results were performed by GraphPad Prism, version 5.00 (GraphPad Software, Inc. San Diego, California, USA, www.graphpad.com).

2.4. SDS-PAGE

Polyacrylamide gel electrophoresis was essentially performed as described by Laemmli [30]. Protein concentration was determined

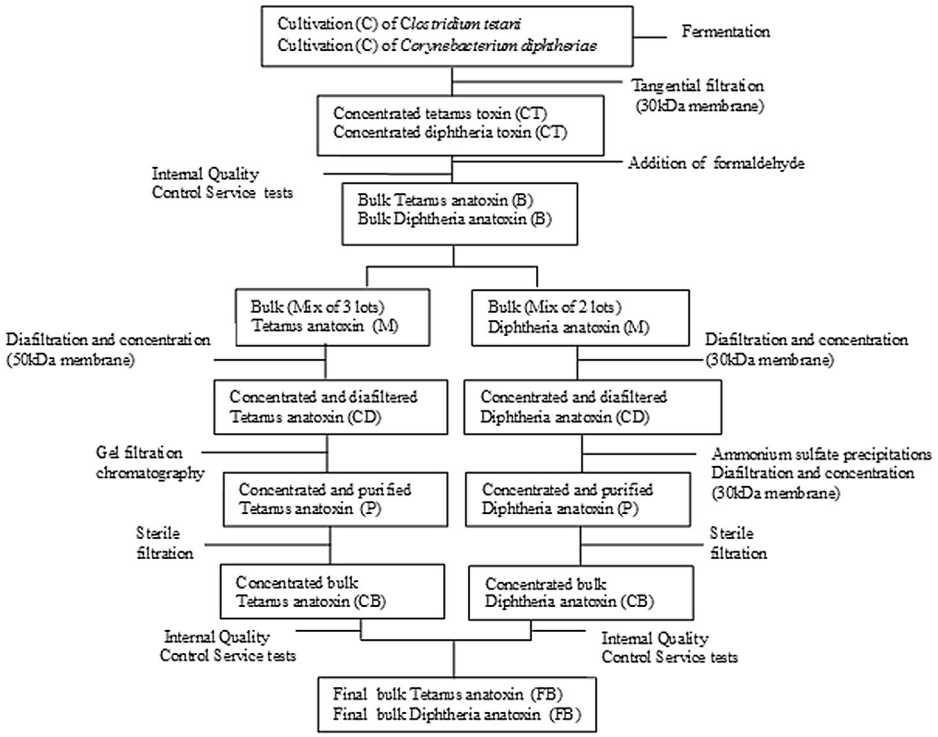

Fig. 1. Flow chart of production processes for tetanus and diphtheria anatoxins in Butantan Institute.