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Original Article

Investigation of the Effect of PEG Detoxification on Diphtheria Vaccine

Hesari, T[1], Tahoori, F[2]*, Nazari, A[3], Salehi Najafabadi, Z[2], Samianifard, M[3], Faramarzi, A[2], Soleimani, M[1]

1. Imam Khomeini International University, Qazvin, Iran
2. Department of Human Bacterial vaccine, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
3. Department of Proteomics-Biochemistry, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

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Corresponding Author: f.tahoori@rvsri.ac.ir

Abstract

Immunization has been considered a successful global health program that saves many persons' lives each year. The vaccines reduce the risk of getting the disease by building immunity in the body. Therefore, the constant availability of essential vaccines is an important factor in community health. One of the most important vaccines is the diphtheria vaccine, which is usually used as *Multivalent diphtheria-tetanus-pertussis* (DTP) combination vaccines. The production of this vaccine takes about 45 days, from the initial bacterial culture to the end of toxin production. However, the production of this vaccine can be optimized in case the production stages are carried out under normal conditions. In this study, a significant amount of impurities was removed after washing with phosphate buffer saline, and the toxin was then purified by Sephadex G-50. In this method, the toxin was concentrated to be stored in a smaller space (this removes the concerns for the provision of a suitable space). Another problem with the diphtheria vaccine is that it is reversible after detoxification of the toxin using formaldehyde. For this reason, it is suggested to use MPEG for detoxification, which will produce more stable covalent bonds between PEG and the first type of amine groups in the toxin chain. Tests were performed to evaluate factors, such as in vivo cytotoxicity, lack of edemas formation, the neutralizing activity of serum from guinea pigs immunized with the diphtheria toxoid inactivated with MPEG, and the immunogenic activity of the purified and modified toxin. Comparison of this PEG detoxification toxoid with the standard toxoid produced in Razi Vaccine and Serum Institution, Karaj, Iran, showed that washing with PBS and purification with Sephadex G-50 was an efficient method. The stability and reversibility of the toxoid approved by MPEG were acceptable. Therefore, the results of animal tests showed that the obtained product was stable and caused no wound or necrosis in the tested animals.

Keywords: Diphtheria vaccine, Detoxification, PEGylation, Toxoid

1. Introduction

Diphtheria is an acute respiratory disease induced by the gram-positive bacterium of Corynebacterium diphtheria (1). Vaccination against the disease has been underway since 1974. The vaccine used for this disease is a toxoid that is obtained from an inactivated toxin produced by formaldehyde (2). For this purpose, culture supernatant is used which contains the desired toxin and impurities, such as unwanted amino acids, peptides, and proteins. It reacts with increasing formaldehyde (WHO approved method) and adjusts the pH of amine positions in all compounds in the

supernatant, and in some cases cross-react with other amino acids (3). Therefore, at the end of detoxification, there will be a mixture of matters with excess formaldehyde residue, which causes local or systemic reactions in the human body (4). Therefore, it is important to find an alternative compound for detoxification of the toxin that does not contain toxic residues.

Polyethylene glycol (PEG) is a synthetic polymer that has been found suitable for medical applications due to its high solubility in aqueous media, biocompatibility, and good tolerance. PEG-conjugated drugs are recognized to be safe for human use by the US Food and Drug Administration (5-7).

PEGylation of proteins has been used for more than three decades to improve the pharmacokinetic properties of protein drugs, drug delivery, imaging, and tissue engineering (5, 8, 9). Several PEG-labeled protein drugs (e.g., the drugs used to treat cancer) are currently in the stage of clinical use (10-12). Following the application of this method, more specific antibodies are formed in the patient's body, which leads to faster treatment and lessens damage to healthy cells.

In the PEGylation process, PEG binding site plays a decisive role in reducing damages to the biological properties of the protein. About half of the drugs on the market still have non-selective PEG from the amino acid lysine side chain, which is a widely used method due to the natural frequency of the amino acid lysine. On the other hand, this frequency leads to non-selective PEG binding and in fact, a mixture of different degrees of PEGylation occurs (13).

This study aimed to provide an alternative method for the current method of diphtheria vaccine production that can reduce the toxic effects of formaldehyde and its irreversibility. For this purpose, the toxin produced was purified with the best chromatographic column, and the immunity of the obtained products was analyzed with standard toxoid produced in Razi Vaccine and Serum Institution (RVSRI), Karaj, Iran.

2. Materials and Methods

2.1. Reagents

The materials used in this study included toxins, toxoids, physiological serum, and distilled water produced by RVSRI, protein marker (SMOBIO Co.), goat anti-guinea pig IgG conjugated with peroxidase (Sigma AP108P, German), BCA reagent test (G-Bioscience Co. USA), G-50 gel, and other compounds, including sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, formalin, glycine, Tris, sulfuric acid, ammonium persulfate, acrylamide, sodium dodecyl sulfate, glycerol, methylenebisacrylamide, bromophenol blue, mercaptoethanol, hydrochloric acid, TEMED, Coomassie Brilliant Blue, silver nitrate, skim milk, and all solvents (Merck and Sigma-Aldrich, German).

2.2. Purification of Toxins

The toxin was purified at two stages. Initially, the toxin was washed with 20 times the volume of phosphate buffer at pH=7.3 using a pump and concentrator (10 kDa filter) (Figure 1). Electrophoresis was used to investigate the amount of impurity. For this test, 20 µg of samples were submitted to polyacrylamide gel electrophoresis in SDS-PAGE (12%) with a discontinuous buffer system.

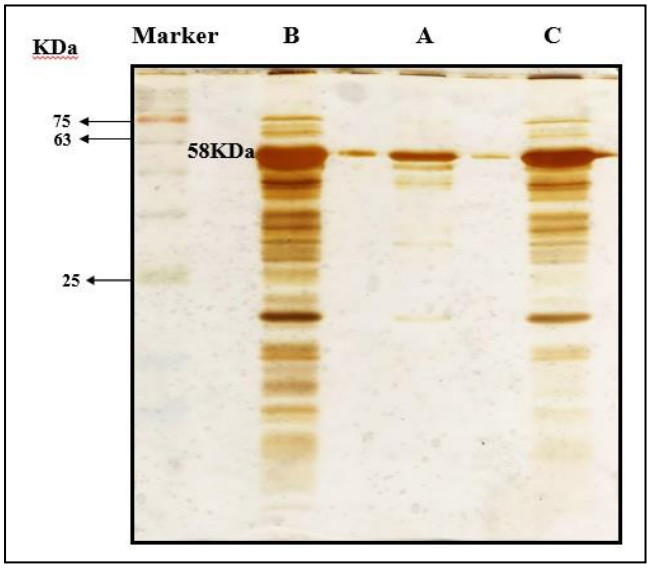

Figure 1. SDS-PAGE analysis of diphtheria toxin (A) Diphtheria toxin provided by RVSRI (B) Concentrated toxin (C) Sample washed with PBS.

At the next stage, the washed toxin was purified using the size exclusion chromatography technique. For this purpose, Sephadex G-50 gel was used, which had the highest efficiency for isolating fractions containing toxin samples from impurities (Figure 2).

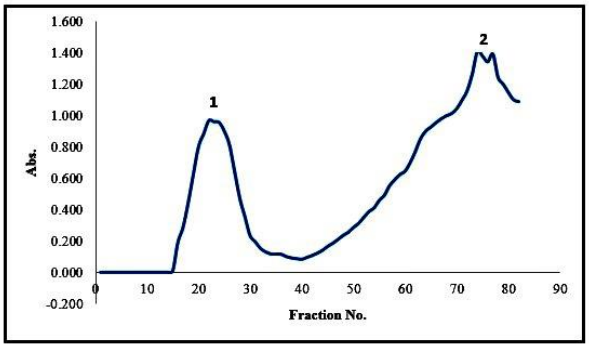

Figure 2. Chromatogram of Diphtheria Toxin using Sephadex G-50

Comparison of the results of toxin electrophoresis and fractions obtained by gel chromatography showed that the main matter was removed from the column at the first peak. This is very important on an industrial scale due to the fact that the column can be easily washed after removing the original sample in the shortest possible time (Figure 3).

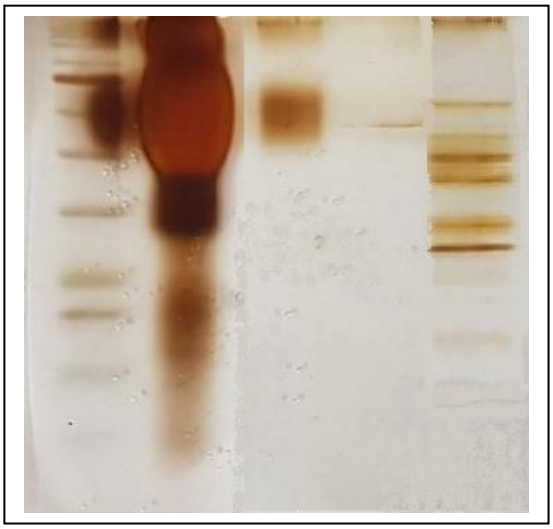

Figure 3. SDS-PAGE analysis of fractions (separation with Sephadex G-50)

2.3. Detoxification Using Formaldehyde

In this method, the pure toxin sample isolated from the column was passed through a 0.2-micron filter, and 40 ml of the sample was poured into sterile containers. According to the WHO standard, 6 ml of formaldehyde should be used for every 1000 ml of a sample. The sample was stirred gently to mix well. The pH of the samples was measured to ensure that they are within the standard range. The pH adjustment was monitored every day for the first three days, and then the monitoring was done every other day until the seventh day, and once a week until the end of the period. In case of pH alterations, pH was adjusted to an acceptable range using one molar soda solution. The samples were shaken gently in a mobile heater at 35 °C for 42 days to convert the toxin to a toxoid.

2.4. Detoxification Using Polyethylene Glycol

The 2,5-dioxypyrrolidine-1-yl acetate compound was synthesized to determine the optimal conditions in terms of time, temperature, and the molar ratio of raw materials (14). The toxin was detoxified by MPEG-SS following the optimization of the reaction conditions.

For the synthesis of 2,5-dioxopyrrolidine-1-yl acetate, acetic anhydride was reacted with N-hydroxysuccinimide in dichloromethane solvent in the presence of DCC catalyst (Figure 4). The conditions of reaction with the purified toxin were optimized, subsequent to the synthesis and purification of the sample.

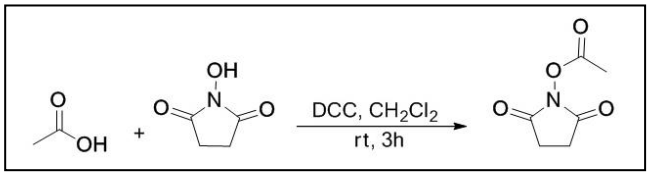

Figure 4. Synthesis of 2,5-dioxipirolidin-1-yl acetate

In this method, purified toxin containing 39 amine groups of the first type (38 amine groups in the lysine side chain and one end amine group) was added to the molar ratio of MPEG-SS and stirred in phosphate buffer solvent (pH=7.4) in ambient temperature for 25 days. At the end of the reaction time, the sample was purified again using a G-50 chromatography column, and the unreacted polyethylene glycol was isolated afterward.

2.5. Immunity Test

In this test, 1 ml of toxoid samples prepared by formaldehyde and PEG methods with 1000 Lf/ml titration was injected subcutaneously into three groups of four guinea pigs. In each group, one guinea pig was placed next to the other pigs as a control. The initial weight range of each piglet was 250-300 g. The weighing was performed on the first day and then weekly, and symptoms were monitored for 42 days.

2.6. Specific Non-Toxicity and Reversal Test

This test was used for the final control of deactivation and non-reversibility assurance. The antigen of the toxoid samples was mixed with physiological serum and aluminum phosphate adjuvant in sterile containers and provided a homogeneous milky suspension.

The toxicity test was performed by diphtheria toxoid (250 Lf) injection (five times the recommended human dose) into guinea pigs with the weight range of 250-300 g. One extra pig was kept as a control in each group. Physiological serum was injected into control pigs in two groups, and no injection was performed on control pigs in other groups. After 20 days from the first injection round, the second round of injection was performed (the same as the first injection) to increase the immune response. During this period, weighting was measured four times (for 42 days) and the symptoms were monitored.

The toxoids were tested in terms of stability by making solution in phosphate buffer saline (PBS) pH=7.4 and stored at 4º C (refrigerator), 25º C, and 37 º C (incubator) for 14 months. Samples were taken at intervals and tested by injection into the backs of guinea pigs. The size and degree of erythema were measured after two days. Lack of toxicity was inferred in case the erythema was not greater than 10 * 10 mm in extent, with no suggestion of induration (15, 16).

2.7. Immunization of Guinea Pigs

At this stage, three groups of 5 guinea pigs in the weight range of 250-300 g were immunized against polyethylene glycol-produced toxoid by formaldehyde. Moreover, one group was immunized as a positive control with diphtheria vaccine produced by RVSRI, and two groups of 3 pigs were tested against aluminum phosphate adjuvant and physiological serum as a negative control.

Three injections were performed on each sample at 2 weeks interval, at a concentration of 40 micrograms of antigens, and blood samples were taken 45 days after the last injection. Animal's weight and health were tested weekly during the immunization period. Subsequent to blood sampling, the serum was isolated using a centrifuge and stored at 20° C in a freezer.

2.8. ELISA Test

The protein concentration of the injected toxoid samples was measured using the BCA test (17). The antigen was diluted by coating buffer and 100 µl was poured into wells. The plate was incubated overnight at 4 ℃ to allow enough proteins to adhere to the bottom of the well. The antigen was then discarded and the wells were washed three times (each time for 3 min) by PBS-T buffer. The buffer blocking protein solution was then used to block the free connection points in the wells and incubated for 1 hour at 37 ℃. Afterward, washing was performed according to the previous stages. The initial antibody was diluted by blocking buffer and added to wells at different concentrations (from high to low) and incubated for 1 hour at 37 ℃.

After washing by PBS-T, 100 µl of secondary antibody (coat anti guinea pig HRP) at the ratio of 1:2000 was added to each well, and the plate was incubated for one hour at 37° C. Then, the wells were washed by phosphate buffer and 50 microliters of BM Blue substrate was added to each well. According to this substrate protocol, to stop the reaction, 100 ml of sulfuric acid solution (1%) was added after 15 min, and

the sample absorption was read by ELISA reader at a wavelength of 405 nm. Figure 5 presents the results of the ELISA test.

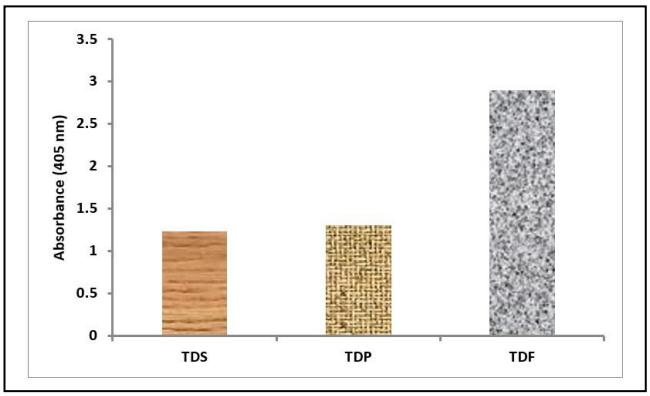

Figure 5. Comparison of the immunogenic activity of formaldehyde-produced toxoid samples and final purification (TDS) of purified toxin samples, MPEG-detoxified (TDP), and purified and detoxified formaldehyde (TDF)

3. Results

Most of the impurities were removed after washing the toxin with phosphate buffer. The washed sample was then passed through the Sephadex G-50 chromatographic gel column, and the obtained toxin protein had a very high purity and was completely clear and colorless. Therefore, a purer toxoid product was obtained for the detoxification phase. The purified toxin was detoxified with formaldehyde and polyethylene glycol separately and the obtained samples were subjected to the necessary tests.

The results of toxicity tests showed that both samples were detoxified, and the results of the irreversibility test indicated the highest stability of the samples detoxified with polyethylene glycol over time. Furthermore, no wounds or necrosis were observed on the animals' bodies following the application of this toxoid.

The ELISA test was used to evaluate the effect of detoxification agents on immunogenicity, and the results showed that samples detoxified with both formaldehyde (TDF) and polyethylene glycol (TDP) were as immunogenic as the standard sample (i.e., the toxoid produced by RVSRI). However, detoxification of purified toxins (TDF samples) with formaldehyde will provide higher immunogenicity.

4. Discussion

Purification is one of the critical stages of vaccine production, as impurity will lead to unwanted biological effects. So far, several methods have been proposed to purify the toxin or diphtheria toxoid to produce higher-yield products at lower costs. In 1983, diphtheria toxin was purified using phenyl sepharose hydrophilic resin chromatography and DEAE-Cellulose ion-exchange chromatography (18). A year later, it was simply presented by gel filtration and deposition with ammonium sulfate (19). The purification path was later improved by changing the static phase of the chromatographic column to Sephacryl S300 and Superdex 75 (20). In 2009, a toxin with a purity of 99% was obtained through a two-stage chromatography using ion exchange resin followed by a hydrophobic resin.

A method used in the last decade involved several stages of deposition by ammonium sulfate, increasing activated carbon, sodium bicarbonate, and Sephacryl S-300 column for industrial scale. In this method, toxoid was achieved with better purity at a lower cost, compared to previous methods.

The common limitations of all these methods included the high price of the given gel, a high number of purification stages, the low flow rate of the mobile phase, a time-consuming purification process, and the low purity of the obtained product. The adoption of the method presented in this study will result in a pure product that can be used on an industrial scale in the shortest possible time at just two stages (washing by phosphate buffer and passing the sample through the Sephadex G-50 column). Moreover, the final products can be concentrated and sterile as far as possible to occupy the least space and have a longer use.

Another major problem with toxoid vaccines is their reversibility over time. Several studies have tried to

address this issue using PEG instead of formaldehyde. The results indicated that the application of this method can increase the stability of the obtained product and decrease the time spent on toxin detoxification.

5. Conclusion

Other than having a shortened purification and detoxification process, the diphtheria toxoid produced in this study was more stable as well. However, the final product in this method showed less immunization, compared to the equivalent amount of formaldehyde detoxified toxin. Therefore, further studies are required to improve PEG toxoid vaccines.

Authors' Contribution

Study concept and design: F. T.
Data acquisition: T. H., A. F. and M. S.
Data analysis and interpretation: F. T. and A. N.
Manuscript drafting: Z. S. and F. T.
Critical revision of the manuscript for important intellectual content: F.T.
Administrative, technical, and material support: Z. S. and F. T.

Conflict of Interest

The authors declare that they have no conflict of interest.

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