Study on Diphtheria Toxin Purification: A New Approach to Prepare Toxoid

Zolfagharian,*H., Mohammadpour, N.
Human Bacterial Vaccines Dept., Razi Vaccine & Serum Research Institute, P.O.Box 11365-1558, Tehran, Iran
Received 26 Oct 2003; accepted 17 Feb 2003

Summary

Two produce a high potency diphtheria toxin two methods of detoxification were applied. In the revised method the toxin was purified before detoxification by formaldehyde–stabilizing agent mixture. In the conventional method the crude toxin was detoxified by formaldehyde alone. In this method culture filtrates contain numerous impurities and metabolites that may cause heterogenous product. The potency and reversibility of toxoid prepared by the revised method were tested and showed desirable immunizing and irreversibility. It possessed a high degree of purity, potency, recovery and showed no tendency to revert to toxin. Our results indicate that the purification index and recovery percentage in the revised method was significantly improved ($p<0.01$) as compared with the conventional method. The percentage of purification 25, recovery 15.6, and potency 9.8 were increased in the revised method.

Key words: diphtheria, toxin, purification, detoxification

Introduction

Corynebacteria are gram-positive rods with a club-shaped appearance. *Corynebacterium diphtheria* (*C.diphtheria*) produces a potent exotoxin that causes diphtheria an acute and fatal bacterial disease in human. Diphtheria toxin is a heat-labile polypeptide (MW 62.000) which causes hemorrhagic and necrotic damages in the mucous membranes of the respiratory tract (Joklik *et al* 1992, Rappuoli 1990).

*Author for correspondence. E-mail:zolfagharian@rvsri.com

The disease can be prevented by active antitoxic immunization. The toxoid is treated with formaldehyde according to the method of Roman (1924). Based on this method immunity to diphtheria can be accqured using nontoxic but highly immunogenic toxoid. Diphtheria vaccine is presently still prepared by Romon method (conventional method). In the large scale production of diphtheria vaccine, an approciate broth culture of a toxigenic strain is prepared and the toxin is harvested by separation from bacterial cells. The crude toxin is immediately detoxified by addition of formaldehyde then the nontoxic crude toxoid is concentated and purified. Linggood *et al* (1963) reported the detoxification procedure with formaldehyde which resulted in higher purity. Their found that purified toxins need additives such as aliphatic diamines containing primary or secondary amino groups to convert into nontoxic products showing no reversion of toxicity upon storage in diluted form at elevated temperature.

In this study based on the findings of linggood *et al*, we were developed a method for the large-scale production of diphtheria toxoid, which derived from purified toxin. In the revised method the toxin was concentrated then crude toxin was purified and finally purified toxin was detoxified by formaldehyde 0.06M , $NaHCO_3$ and stabilizing agent. Also, the purity, potency and recovery of toxoid prepared by revised and conventional methods were studied.

Materials and Methods

Toxin. A filtrate of broth culture of a hypertoxinogenic *C.diphtheria* strain no PW8 was used. The filtrate solution contained toxins (Mirchamsy 1987,W.H.O 1973).

Detoxification and purification of diphtheria toxin. Two methods of detoxification were used. In conventional method the diphtheria toxin were detoxified by 0.07M of formaldehyde (Sigma) with well shake. pH was checked and adjusted to 7.2 during an incubation period of six weeks at 37°C. After detoxification the crude toxoid was

concentrated by ultrafiltration (amicon apparatus with cartridge type HIOPIO-20, cut-off 10000, USA) (Robb *et al* 1970, Rappuoli 1990) and fractional precipitated with 24-44%w/v ammonium sulphate (Fluka). The ammonium sulphate precipitate was dialyzed (cut off 3000) against 0.85%w/v, NaCl (Merck), refractionated by sephadex G-50 (Sigma) and sterilized by membrane filtration (Millipore) (Harris & Angal 1989, W.H.O 1977, Rappuoli 1990). In revised method, the diphtheria toxin were concentrated by ultrafilter, fractionated by ammonium sulphate (26-44%w/v), purified by gel chromatography (sephadex G-50) and finally detoxified (Rappuoli 1990). The purified toxin was diluted to approximately 500Lf/ml and detoxified in the present of 0.06M formaldehyde, 0.06M $NaHCO_3$ (Merck) and stabilizing agent at 22°C for 4-5 weeks and pH adjusted to 7.2.

Antigenic purity. Antigen titeration was performed by Lf determination using diphtheria antitoxin (standardized against the WHO standard diphtheria antitoxin). Protein nitrogen levels were estimated upon trichloroacetic acid-precipitable material by the micro-Kjeldahl procedure. The purity of the various toxoids was expressed in terms of Lf per mg (U.S.P 2000).

Antigenic potency. Groups of 10 guinea pigs (250-300gr) obtained from Razi Institute were injected subcutaneously with 1.0ml of three different concentration of the toxoid. After three weeks the animals were challenged by the subcutaneously injection of 10MLD (minimal lethal dose) of a standard diphtheria toxin. The ED50 of antigen calculated from log dose-response curve by probbit analysis (W.H.O 1973).The ED50 is the level of antigen, which protects 50% of the animals for 7 days after challenge.

Toxicity and reversal tests. The toxicity test was performed by injection of 250Lf diphtheria toxoid (5 times the recommended human dose) into each of five guinea pig (300-400gr). The animals were observed daily for 35 days for any evidence of diphtheria toxicity including necrosis, weight loss, paralysis and death. The toxoids were tested for stability by making solutions containing 50Lf/ml in phosphate-

buffered saline (pH7.3) and storing these at 4, 25, and 34°C for periods of up to 13 months. Samples were taken at intervals and tested by injecting intracutaneously 0.1 and 0.2ml (equivalent to 5 and 10Lf) into the shaved backs of mature guinea pigs. The size and degree of erythema were measured after 48h. Lack of toxicity was inferred if the erythema was not greater than 10×10mm in extent, with no suggestion of induration. Any sample showing more severe reactions was suspected of containing free toxin and was placed on the full toxicity test (W.H.O 1973).

Protein nitrogen determination. The protein nitrogen was determined by measuring the nitrogen content, in every steps of the purification process by the micro–kjeldahl method (U.S.P 2000).

Results

In this study three batches of diphtheria culture were selected. Specification of purified toxins were evaluated by conventional and revised methods (Table 1).

Table 1. *Specification of purified toxoid prepared by conventional and revised methods*

Batch No.	Lf/mg P.N		P.I		R%		P%		ED50	
	Con.	Rev.	Con.	Rev.	Con.	Rev.	Con.	Rev.	Con.	Rev.
1	1523	2445.2	2.60	4.36	57.00	72.00	51.80	86.70	85.00	70.00
2	1764	2448.9	3.00	4.27	53.00	75.60	66.90	74.30	80.00	60.00
3	1555	2567.5	3.00	5.00	60.50	69.60	52.80	87.4	85.00	70.00
Mean	1614	2487.2	2.90	4.54	56.80	72.40	57.10	82.80	83.30	66.60
S.D	±130	±56.75	±0.23	±0.32	±3.72	±2.46	±8.4	±6.01	±3.72	±4.70

Con=Conventional, Rev=Revised, Lf=Lime of flocculation, P.N=Protein Nitrogen, P.I=Purification Index, R=Recovery, P=Purity

The prepared toxoids were analysed by gel electrophoresis (SDS-PAGE) (Fgure 1). As it is shown in the figure in revised method the toxoid has a single band with 60kD molecular weight (lane5), whereas in other method it has a band in 60kD (lane7) and few extra bands with 10-30kD.

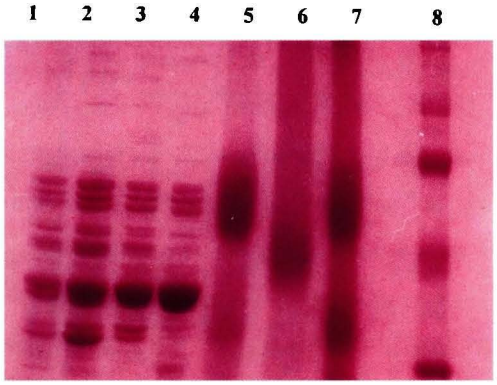

Figure 1. *Electrophoresis pattern (SDS-PAGE). Lane 1-4: crude diphtheria toxin, lane 5: diphtheria toxin after ammonium sulfhate precipitation, lane 6: diphtheria toxin prepared by revised method, lane 7: diphtheria toxin prepared by conventional method and lane 8: protein marker*

In order to make more purification on concentrated diphtheria toxin by revised method, the salting out procedure was used to isolate specific and nonspecific proteins. The ammonium sulphate concentration and production yield of toxin in different steps are showed in table 2.

Table 2. *Ammonium sulphate concentration in first and second precipitation process in revised method*

Batch No.	V (ml)	Lf $(ml)^{-1}$	First precipitation				Second precipitation			
			Ammonium sulphate%	V (ml)	Lf $(ml)^{-1}$	R%	Ammonium sulphate%	V (ml)	Lf $(ml)^{-1}$	R%
1	750	700	26.00	750	690	98.60	18.00	38.0	12000	89.00
2	750	530	27.00	750	500	94.30	17.00	31.0	11000	91.00
3	750	500	27.50	750	490	98.00	15.50	29.40	11000	88.00

In the salting out process, specific protein was separated and desalted by dialysis method. In the final step of purification process the desalted protein refractionated by gel filtration. The specification of both proteins were evaluated (Table 3).

Table 3. *Specification of desalted specific protein and highly purified diphtheria toxin in the revised method*

Batch No.	Desalted specific protein						highly purified diphtheria toxin				
	V (ml)	Lf $(ml)^{-1}$	mg P.N $(ml)^{-1}$	Lf/mg P.N	P.I	R%	Lf/mg P.N	P.I	R%	MLD	P% If=2.5 mg
1	75	55000	1.50	3669.7	3.6	78.5	2545.3	4.36	72.0	5×10^3	86.7
2	65	5000	1.40	3452.0	3.0	82.4	2448.9	4.27	75.6	1×10	74.3
3	68	4500	2.12	2112.7	4.0	78.0	2567.5	5.00	69.6	1×10^2	87.4

In the revised method two ways were used for detoxification of purified diphtheria toxin, detoxification with formaldehyde and detoxification with formaldehyde-stabilizing agent. The toxicity test showed that the detoxification was completed after 30 days in both ways. Our results showed that toxoid, which prepared by formaldehyde-stabilizing agent showed desirable stability and was irrversible, whereas other treated toxoid was unstable and graduatly reversed to toxin form.

Potency testing. The potency of toxoid, which prepared by revised method was measured on guine pigs. For challenge a volume of 10MLD of standared diphtheria toxin was used and the log dose-response curve of toxoid was plotted. Our results showed that ED50 of toxoid was 66.6Lf.

Discussion

In the fermentation process of *C.diphtheria*, exotoxins and other metabolites release in the broth culture. In the conventional method some covalent bands may create between nonspecific proteins, which are present in the broth or with toxoids.These reactions lead to a cross-linkage between an ε- amino group of lysine and a second amino group of histidine, and a tyrosine or a tryptophan through a stable methylene bridge (-CH2-). These reactions can occur between amino acids of the same toxin

molecule, and resulting in internal cross–linking of the protein, between two toxin molecules resulting in dimerization and/or between a small peptide present in the medium and a toxin molecule. These proteins formed due to these reactions may remain with toxoids till end of purification process and it is very difficult to separate them. These impurities associated with diphtheria vaccine and can cause delayed reactions in vaccinated children and adults. An easy way to avoid most of these impurities is to purify diphtheria toxin before detoxification. In the revised method the toxin purified then detoxified by formaldehyde-stabilizer. So, the possibility of creation of above mentioned intra and inter molecular reactions reduced to minimum amount, and the produced vaccine cannot cause allergic side effects (Rappuoli 1990).

SDS-PAGE pattern of diphtheria toxoid which prepared by two methods showed in figure 1. As it is showed the toxoids which prepared by revised method has a single band with 60kD molecular weight, whereas the other toxoid in addition to this band, has few more bands with 10 to 30kD indicate imperities lead to decrease purification index and purity of toxoid.

It seems that peroxidases which present in culture medium can effect on toxins and converted it to toxoid graduately during the purification process. Formaldehyde reactions can take place in two steps. The first reaction involves mainly the ε- amino group of lysine and is rapid and fully reversible according to below reaction:

$$R\text{-}NH_2+CH_2O \leftrightarrow R\text{-}NH\text{-}CH_2OH+H \leftrightarrow R\text{-}NH{=}CH_2+H_2O$$

Whereas the second reaction occurs much more slowly and involves the reaction one of the above unstable products, with a second molecule containing an amino group, an imidazole rings or a phenol ring, and due to these reactions the stable compound were formed (Rappuoli 1990, Paliwal & London 1996). The comparison of the conformation, hydrophobicity, and model membrane interactions of diphtheria toxin to those of formaldehyde stabilization of native conformation inhibits changes that allow membrane insertion (Paliwal & London 1996).

For prevention of toxoid reversibility, various stabilizer were evaluated and suitable stabilizer agent were selected. In the presence of stabilizer the following reaction occurred:

R-NH2+CH20+stabilizer→R-NH-CH2-stabilizer

Due to above reactions between formaldehyde, toxin and stabilizer resulting in methylene bridge and lead to a stable product, which is irreversible. The potency and reversibility test of toxoid treated formaldehyde-stabilizing agent showed desirable immunizing activity an irreversibility of toxoid (Rappuoli 1990).

In the present study, it is interesting to find that those toxoids contain stabilizer did not show any differences in immunogenicity from those toxoids which did not contain stabilizer. Our results was inagreement with other studies (Stainer 1967, Rappuoli 1990). Although stabilizer did not appear to affect the antigenicity of diphtheria toxoid, it had a profound effect on the stability of the diphtheria toxoid. Our findings confirmed by Stainer (1967). Linggood *et al* (1963) and Scheibel *et al* (1965) demonstrated that when purified diphtheria toxins were detoxified with formaldehyde, reversion to toxin occurred, and when stabilizer–formaldehyde mixture were added to the toxin a stable nonreversing toxoid was formed. It was shown that if stabilizer was included in the detoxification solution the toxoids obtained were stable and immunogenic (Pappen heimer 1984, Rappuoli 1990). Frech *et al* (2000) studied on diphtheria vaccine. They suggested that the specific purity, immunogenicity of purified toxoid obtained from revised method was highly significant as compared with toxoid gained from conventional method. Our results also showed that the treatment of diphtheria toxin with formaldehyde-stabilizing mixture resulted in a better detoxification process and finaly the toxoids obtained were stable, immunogenic, and highly potent. Statistically *t* values indicated that purification index and recovery in the revised method is highly significant as compared with other method. Our results show percentage of purification 25%, recovery 15.6% and potency 9.8% increase in the revised method.

It can be concluded that the diphtheria antigen prepared by revised method has following advantages: higher purity (low hypersensivity reaction), irreversibility to toxin (low toxic reaction), and higher potency (high immunogenicity with low dose).

References

Frech,C., Hilbert,.A.K., Hartmann, G., Mayer, K., Sauer, T. and Bolgiano, B. (2000). Physicochemical analysis of purified diphtheria toxoids. *Development Biology Basel Karger* 103:205-215.

Harris, E.L, Angal, S. (1989). *Protein Purification Method.* (2nd edn.), Oxford University Press.

Joklik, K., Willet, P., Amos, D.B. and Wilfert, C.M. (1992). Corynebacterium. In: *Zinsser Microbiology* (2th edn.), Pp:487-496. Appleton and Lange Press.

Linggood. F.V., Sterens, M., Fulthorpe, A.J., Werewod, A.J. and Pope, C.G. (1963). The toxoiding of purified diphtheria toxin. *British Journal of Experimental Pathology* 44:177-188

Mirchamsy, H. (1987). *Prevention and Treatment with Diphtheria Vaccine and Serum.* Pp:147-156. Tehran University Press (In Persian).

Paliwal, R., London, E. (1996). Comparison of the conformation, hydrophobicity, and model membrane interactions of diphtheria toxin to those of formaldehyde-treated toxin (diphtheria toxoid): Formaldehyde stabilization of the native conformation inhibits changes that allow membrane insertion. *Biochemistry Journal* 35:2374-2379.

Pappenheimer, A.M.Jr. (1984). Diphtheria. In: R. Germanier (Ed.), *Batcterial Vaccines.* New York:Academic Press.

Ramon, G. (1924). Sur la toxine etsur l'anatoxine diphtheriques. *Annual of Institute Pasteur* 38:1-5.

Rappuoli, R. (1990). New and improved vaccines against diphtheria and tetanus. In: *New Generation Vaccine.* Pp:251-268. Marcel Dakker, INC.

Robb, L.A., Stainer, D.W. and Scholte, M.J. (1970). Preparation and properties of diphtheria toxoids in submerged culture: A revised method for the purification of diphtheria toxin. *Canadian Journal of Microbiology* 16:639-643.

Scheibel, E.I., Christensen, P.E. (1965). Irreversible detoxification of purified diphtheria toxin. *Acta Pathology Microbiology Scandinavia* 65:117-128.

Stainer, D.W. (1967). Preparation and properties of diphtheria toxoids in submerged culture. II. Purification, detoxification, antigenicity, and stability. *Canadian Journal of Microbiology* 14:327-330.

United State Pharmacopoeia. (2000). Handbook of USP (24) National formulary (19), USP convention, Inc. 12601 Twinbrook, Parkway, Rockvile, MD.

World Health Organization Mondiale DE LA Sante. (1977). *Manual for the Production and Control of Vaccines.*