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Production, Optimization of Detoxification and Ammonium Sulphate Precipitation of Ultrafiltered Tetanus Toxin

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Abstract

The present project work was undertaken with the aim of producing a safe & potent tetanus toxin by static culture method, optimization of detoxification process for the conversion of toxin to toxoid and its purification by ammonium sulphate precipitation. Tetanus toxin can be converted into a safe, highly potent and irreversibly detoxified vaccine either using the conventional method in which the crude toxin is chemically detoxified and subsequently purified or by formalization of the purified toxin in the presence of artificial matrix. In this study, we evaluated another approach for preparation of tetanus toxoid by concentrating and partially purifying the toxin followed by detoxification with formaldehyde-stabilizing agent mixture. The increase in purity was observed after ultrafiltration of crude tetanus toxin. So ultra-filtration is useful for the partial purification of tetanus toxin. The toxin was detoxified completely within 4 weeks of incubation both in the presence and absence of stabilizing agents. The pH and lf/ml value of the toxin decreased during 6 weeks of incubation at 37°C of the toxoid. According to the preliminary curve, the tetanus toxoid was found to precipitate between 12 to 24% concentration of ammonium sulphate.

Key Words: Tetanus toxoid; Static culture; Detoxification; Ammonium sulphate precipitation ; Formalization; Ultrafiltration; Lf/ml

Introduction

Vaccination is regarded as one of the most beneficial biopharmaceutical interventions, due to its ability to induce protection against infectious diseases through targeted activation of the immune system. Tetanus is an acute and often highly fatal disease of humans caused by exotoxins produced by the bacterium *Cl.tetani*. *Cl. tetani* produces two exotoxins- tetanolysin and tetanospasmin. The function of tetanolysin is not known with certainty. Tetanospasmin is a neurotoxin and causes the clinical manifestations of tetanus [1-4]. Tetanus occurs almost exclusively in persons who are inadequately immunized. Tetanus toxoid is one of the most immunogenic antigens available for the protection against an infectious disease in the world. Its use has markedly decreased the demand of tetanus antitoxin, but in the developing world much needs to be done to its availability. Since tetanus can be easily controlled by immunization with tetanus vaccines prepared from purified tetanus toxoid, thus there is a great demand of purified tetanus toxoid of good quality and high antigenic value. It is prepared from tetanus toxin, which causes the clinical manifestations of the disease in man. The toxin which appears in a culture of toxigenic *Clostridium tetani* strain is converted by formaldehyde into a nontoxic but still immunogenic tetanus toxoid. The classical vaccine is produced via a number of steps: cultivation of *Clostridium tetani* 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 strain of bacterium, the composition of the culture medium and the growth culture conditions are important in obtaining a high yield of toxin and also in ensuring that the subsequent treatment of toxin results in the production of a safe vaccine of high immunogenicity. Optimization of aeration, incubation time and temperature for sterilization etc. are few factors which are responsible for the production of safe and potent tetanus toxoid [5-7]. It has been known since the early years of this century that treatment with formaldehyde will convert certain powerful bacterial toxins into non toxic products that can be used for active immunization. Formol toxoids were not used in the field until the 1920's when the work of Glenny and of Ramon led to the immunization of children against diphtheria, and the work of Descombey [8] to the immunization of animals against tetanus.

The treatment with formaldehyde has a large effect on the structure of the antigen, and affects the toxicity, antigenicity, immunogenicity and stability of the protein. The formaldehyde reaction yields intramolecular cross links, which stabilize the protein structure, but also causes the loss of some epitopes. In general, the toxoids remain very immunogenic, and induce a protective response. Six different types of amino acid residues

and the N-terminal amino acid of a protein are reactive to formaldehyde. Thus, the quality of tetanus 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 [9]. During inactivation, formaldehyde reacts first with amino groups; in the second step, cross-links are formed between the reaction product and several other amino acids. 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. To eliminate the risk of cross-linking foreign proteins to toxoids in an attempt to reduce the frequency of adverse reactions in vaccination programmes, it is preferable to purify toxins before treatment with formaldehyde [10].

Tetanus toxoid of high purity can be prepared either by detoxification of purified toxin or by detoxification of crude toxin followed by purification of toxoid. Purification of tetanus toxoid is necessary in order to eliminate constituents of the growth medium and metabolites which tend to provoke undesirable reaction, and to provide a purified concentrated toxoid which can be incorporated into multivalent vaccines such as DPT and DT vaccines. Ammonium sulphate is by far the salt which is most frequently used by toxoid producing laboratories all over the world including India for both toxoid concentration and fractionation. The effectiveness of ammonium sulphate is higher than other salts due to its extreme solubility (700 gm per litre) [11]. Taking into account the importance of detoxification process and purification step in the production of the tetanus toxoid, the present study has been undertaken to compare the two approaches for the purification and detoxification of tetanus toxin and to optimize the conditions for the detoxification and purification of concentrated tetanus toxoid.

Materials and Methods

The reagents used in the present study were of analytical grade/ LR grade.

Production strain

Harvard strain of *Clostridium tetani* no. 49205 originally obtained from RIVMs Netherlands was used for the production of toxin. A secondary seed in lyophilized state was prepared from the above and routinely used for vaccine production at Triple Vaccine Division, Central Research Institute, Kasauli, HP, India.

Revival of the production strain

Lyophilized culture of *C. tetani* was reconstituted aseptically into 1 ml of fluid thioglycollate medium(FTM).

Preparation of seed culture

Revived culture was transferred to 100 ml of Fluid thioglycollate medium. The medium was incubated at 35°C for 48 hours. The pure growth was further subcultured in heart infusion glucose broth medium (HIGB), inoculated tubes were incubated in the McIntosh Fildes Jar at 35°C for 24 hours in reduced environment.

Production of tetanus toxin by Static Culture Method

The toxin was prepared by static culture method [12, 13] in sterile 20 L stainless steel pots. 14 L of Muller and Miller III medium was distributed in each stainless steel pot and sterilized at 118°C for 20min. 0.1% of pure seed culture (which was already checked for its purity) was inoculated in every stainless steel pot under aseptic conditions. All these inoculated pots were incubated at 35^0C immediately for 7-8 days.

Harvesting of Toxin

The toxin was harvested on 7th day. Before harvesting all containers were checked for purity, antigenic value in terms of Lf/ml, pH and then Seitz filtered through asbestos filter pads k5 and EKS (Pore size k-5> 0.45 µm and EKS>0.22 µm). The container showing Lf/ml value <20 were discarded and not taken for harvesting.

Determination of antigenic content

Toxin was assayed in terms of Lf/ml by Ramon's flocculation test.[14] The correct zone of flocculation was further confirmed by toxin-antitoxin neutralization test in mice (MTV).

Detoxification of the Toxin

Two methods of detoxification were used. In conventional method the tetanus toxin was detoxified by 0.45% formaldehyde (Loba Chemie) and incubated for a period of six weeks at 36°C. After detoxification the crude toxoid was concentrated by Ultrafiltration system(Millipore India Ltd.) and purified by ammonium salt fractional precipitation.

For the production of a series of experimental tetanus toxoids by adapted method, the toxin-containing culture fluid was concentrated by Ultrafiltration (Molecular weight cut off value: 10 kDa) to remove medium components of low-molecular weight, such as amino acids and peptides. After Ultrafiltration, the toxin preparations were sterile filtered through 0.22 µm cartridge filter in aseptic conditions. A glycine solution of 2.0 M was added to concentrated toxin to a final concentration of 20, 40, 60, 80 and 100mM. To start the inactivation reaction, a diluted formaldehyde solution (Loba Chemie) of 2.0 M was added to a final concentration equimolar to that of glycine (Merck). The reaction mixtures were kept at room temperature for 3 days and the pH was adjusted to 7.6 before incubation at 37°C. Each reaction mixture was tested for residual toxicity at 3rd and 4th week.

Quality control tests

Antigenic purity[12,13]: Antigen content was estimated by Ramon flocculation test using in house working standard of tetanus antitoxin for flocculation(TATF) (standardized against the WHO standard tetanus antitoxin).Protein nitrogen levels were estimated upon trichloroacetic acid precipitated material by the Kjeldahl's method. The purity of the various toxoids was expressed in terms of Lf/ mg PN_2.

Estimation of maximal toxin value (M.T.V.)

MTV was assayed by mixing constant amount of toxin (1ml) with increasing amounts of antitoxin in the same manner as in the flocculation reaction. The tubes were well mixed and kept at room temperature for one hour and 0.5ml of each mixture was injected subcutaneously into three mice. The mice were observed for four days. [12,13]

The mixture which just failed to produce symptoms of clinical tetanus in mice contained equivalent amounts of the toxin and antitoxin which had neutralized each other and was considered as maximal toxin value units per ml.

Determination of minimum lethal dose (MLD)

Tetanus toxin was diluted in peptone water to make a concentration range from 1/1000000, 1/2000000, 1/4000000 and 1/8000000. Each mixture (0.5 ml) was injected in two mice subcutaneously. Mice were observed for four day. The MLD is that amount of toxin which kills majority in four days [12,13].

Sterility Test on the Toxin

It was put up from each bottle containing the seitz filtered toxin. Samples were withdrawn aseptically with a sterile syringe and two bottles of thioglycollate medium were inoculated for each bottle of toxin. About 1 ml of inoculum was added in each bottle and bottles were incubated at 35º C for 14 days. These were observed daily and the test was passed when found sterile at the expiry of the period of observation

Detoxification test

Formalized toxin (1 ml) was injected subcutaneously into two mice each having 14 to 16 g of body weight after 3rd and 4th week. Mice were observed for 10 days for absence of tetanus symptoms12,13].

Determination of Hydrogen ion concentration (pH)

The pH meter electrode was standardized with Standard buffer solution at 4.0, 7.0 and 9.2. Further it was ensured that temperature knob was at its ambient temperature. Electrode was rinsed with distilled water and immersed in test solution. pH displayed on the display screen of pH meter was recorded.

Partial purification of crude tetanus toxin

Conversion of crude tetanus toxin into a toxoid by formalization was the basis for the production of a safe and effective prophylaxis against tetanus. These first reasonably crude vaccines were soon improved by the introduction of purification methods such as fractional precipitation by ammonium salts, ultrafiltration, dialysis, gel filtration and more in recent time's chromatography[15]. Even with considerable purification efforts the purity of toxoids is only in the range of 60-70%. The parentral administration of tetanus toxoid is occasionally accompanied by undesirable side reactions [16] In addition to the specific antigen, crude tetanus toxoid contains a variety of impurities like residual unutilized amino acids mineral salts, non specific proteins polysaccharides and lipid complexes. These substances obtain either from the culture medium or from metabolism of growing bacteria or both.

Tetanus toxoid of high purity can be prepared either by detoxification of purified toxin or by detoxification of crude toxin followed by purification of toxoid. The toxoid obtained by the first method is homogeneous, highly purified but its disadvantage is that toxoid can revert back to toxin if toxoiding is not done in the presence of stabilizing agent. The second method yields a heterogeneous product with toxoid and formalinized bacterial proteins covalently cross linked together. This toxoid is very stale but lack purity and such toxoid can cause delayed reactions in children and adults. Several workers have supported the concept of purifying the toxin before detoxification. In the absence of associated proteins, the irreversible conversion of purified toxins into toxoids requires the addition of stabilizing agents.[17,18]

Purification of tetanus toxoid is necessary in order to eliminate constituents of the growth medium and metabolites which tends to provoke undesirable reactions and to provide a purified concentrated toxoid which can be incorporated into multivalent vaccines such as DPT and DT vaccines [16]. The purity was estimated for the three batches before and after concentration. The increase in purity was observed for all the three batches of concentrated and ultrafiltered toxin. Thus the ultrafiltration of crude toxins can be handy for the partial purification of toxins after removal of associated media components to a reasonable extent.

Fractional precipitation with ammonium sulphate

Several workers purified tetanus toxoid or toxin by using ammonium sulphate [19]. The optimum concentrations of ammonium sulphate for precipitating impurities and specific toxoid were determined by a pilot experiment. In this study, Batch C with formalin at 20mM concentration, having Lf/ml 240 and purity of 1041 Lf/mg was taken for ammonium sulphate precipitation. For this purpose the pH of the concentrated toxoid was adjusted to 7. 10 ml of toxoid was taken into each of 10 tubes. To these tubes increasing amount of recrysatllized solid ammonium sulphate was added, to give the following concentrations: 8,10,12,14,16,18,20,22,26%.The tubes were shaken to dissolve the ammonium sulfate and left at room temperature overnight. The next day the tubes were centrifuged, the supernatant was decanted and the precipitate was dissolved in deionized water and the volume was made upto 10ml. The Lf value of each precipitate was determined .A graph is plotted with concentrations of ammonium sulphate on the X-axis and the Lf values of the corresponding precipitate on the Y-axis.

Results and Discussion

In this study, three batches of crude tetanus toxin were produced by static culture method in sterile stainless steel pots (working capacity of 14 liters), using a highly toxigenic strain of *Cl. tetani* (Harvard strain no. 49205) and subjected to various in process quality controls tests including sterility, determination of antigenic content, minimum lethal dose (MLD), maximal toxin value (MTV), and specific purity. The maximum toxin yield was obtained on 7th day and the antigenic content varied between 25 and 40 Lf/ml. The Kf for all the three batches was found to be same.

Maximum toxin value of the two batches were determined to check for the correct flocculation zone since tetanus toxin has a tendency to show multiple zone of flocculation. The MTV estimated for the tetanus toxin batches A, B and C was 30, 40, and 30 respectively (Table No.1). The MLD was estimated randomly for batch A and was found be 4 Million (Table no.2).

The specific purity of crude tetanus toxins batches ranged between 750 and 950 Lf/mgPN_2.

Table No.1: Determination of MTV

Batch no	Units(in lf\ml)	Day of observation 1 2 3 4	Result
A	10	d/d	
	20	t/t tt/tt tt/tt ttt/ttt	30
	30	√ √ √	
B	20	tt/tt d/d	
	30	t/t tt/tt d/d	40
	40	√ √ √ √	
C	10	d/d	
	20	√/t t/ttt ttt/d ttt/d	30
	30	√ √ √ √	

d- Death, (t, tt, ttt - Degree of symptom), √- no symptoms of tetanus

Table No. 2: Determination of MLD

	Dilution	Day of observation 1 2 3 4	Result
Batch no. A	1M	t/t ttt/ttt d/d	4 million
	2M	t/t ttt/ttt d/d	
	4M	t/t t/t tt/tt ttt/d	
	8M	t/ √ t/t tt/t tt/ tt	

Partial purification of crude tetanus toxin

Each individual batch of crude tetanus toxin was concentrated and partially purified by ultrafiltration process using membrane cassettes (cut off 10 KD).The lf/ml, specific purity, and % recovery was estimated after concentration and partial purification. The increase in Lf/ml perfectly correlated with extent of concentration and the percent recovery was roughly ≤95%. The specific purity was estimated for the three batches after ultrafiltration and increase in specific purity was observed for all the three batches ranging from 930 to 1430 Lf/mg PN_2. There was no change in the Kf value of the concentrated toxins and remained same as earlier i.e. 5 minutes (Table no.3).

Table No. 3: Specifications of crude and partially purified tetanus toxin

B. no.	Vol. (in liters)	Lf/ml	Kf (in min)	Purity (lf/mg PN2)	Vo l. (after concentration)	Lf/ml	Kf (in min)	Purity (lf/mg PN2)	% recovery
A	6.5	26	5min	753	1.9	84	5min	933	94.44
B	12.5	40	5min	883	1	480	5min	1428	96
C	21.5	26	5min	947.12	2.25	240	5min	1041	96.6

Detoxification of the toxin

The samples were taken out from each bottle after 3rd and 4th week and were tested for residual toxicity. The samples were injected in mice and observed for 10 days. The samples which were taken after 3rd week were found to be detoxified except Batch A with formalin at 20mM, Batch B with glycine and formalin at 20mM as well as with formalin(without glycine) at 20mM, Batch C with glycine and formalin at 20mM.

The toxin was detoxified completely within 4 weeks of incubation at 37^{0}C when equimolar quantities of formalin and glycine were used between 20mM to 100mM. The same results were obtained when formalin was used alone (without glycine) between 20mM to 100mM.

Changes in Lf/ml and pH after detoxification

The pH and Lf/ml value decreases after 6 weeks incubation at 35^{0}C of the toxoid. The mean loss in Lf/ml for batches A, B, C without glycine was 7.73%, 11.54% and 11.62%. While the mean loss in Lf/ml for batch B and C with glycine was 9.22% and 8.5%.There was decrease in the pH towards acidic side in all cases.

Table no. 4: Comparisons of toxoids before and after detoxification in terms of Lf/ml and pH

	Batch no.	Lf/ml before detoxification	Lf/ml after detoxification (6 weeks)	% decrease in lf/ml	pH before detoxification	pH after detoxification (4th week)
A(with formalin)	20mM	84	80	4.76	7.3	6.96
	40mM	84	80	4.76	7.2	6.48
	60mM	84	76	9.5	7.0	6.40
	80mM	84	74	11.9	7.0	5.96
B(with glycine)	20mM	480	448	6.6	7.3	6.81
	40mM	480	448	6.6	7.1	6.42
	60mM	480	432	10	7.0	6.20
	80mM	480	428	10.8	7.0	6.18
	100mM	480	422	12	6.8	6.12
B (without glycine)	20mM	480	432	10	7.5	6.8
	40mM	480	428	10.8	7.0	6.44
	60mM	480	422	12	6.9	6.42
	80mM	480	422	12	6.8	5.74
	100mM	480	418	12.9	6.7	5.68
C(with glycine)	20mM	240	226	5.83	7.5	7.0
	40mM	240	220	8.3	7.1	6.56
	60mM	240	220	8.3	7.0	6.48
	100mM	240	212	11.6	7.0	6.04
C(with out glycine)	20mM	240	216	10	7.2	6.82
	40mM	240	220	8.3	7.0	6.53
	60mM	240	212	11.6	6.8	5.9
	80mM	240	200	16.6	6.7	5.7

Purification of tetanus toxoid by Fractional salt precipitation

The Lf value of each precipitate was determined .A graph is plotted with concentrations of ammonium sulphate on the X-axis and the Lf values of the corresponding precipitate on the Y-axis. On obtaining the preliminary sigmoid curve, we found that the salt concentration between 12-26% was most effective for the purification of the toxoid (Table no.5).

Table 5: Fractional precipitation by ammonium sulphate precipitation

Tube No.	Ammonium salt concentration(w/v)	Lf/ml	Lf/ml (%)
1	8	10	4.17
2	10	12	5
3	12	80	33.33
4	14	102	42.5
5	16	132	55
6	18	144	60
7	20	160	66.67
8	22	168	70
9	24	162	67.5
10	26	156	65

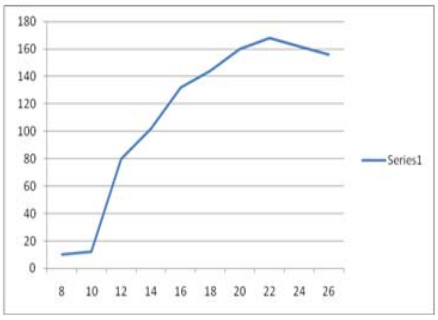

Figure: 1: Preliminary sigmoid curve (a graph was plotted with concentrations of ammonium sulphate on the X-axis and the Lf values of the corresponding precipitate on the Y-axis).

Summary and Conclusion

Muller and Miller's medium is one of the most acceptable and widely used medium for the production of tetanus toxin. All the growth factors in the Muller Miller media are found to be essential. The time and temperature of sterilization and cooling of the media are critical factors which require care and attention in order to have better yield of the toxin [20].

1. The toxin was detoxified completely within 4 weeks of incubation in both conditions (i.e. with or without glycine). Irrespective of antigenic value (Lf/ml) of tetanus toxin; all the experimental batches got converted into toxoid within same time period.
2. The pH and lf/ml value of the toxin decreased during 6 weeks of incubation at 35^0C of the toxoid. During this process there occurs a steady change in the ratio of toxin to toxoid, until no detectable toxin is present. The optimal

pH level for toxin is different from that for toxoid; it would follow that at first the best reaction should be one to favour stability of toxin and later of toxoid. The shift of pH towards acidic side is favorable for stability of toxoid. The antigenic loss was maximal when higher concentration of formalin was used, therefore moderate concentrations of formalin can be used to overcome the antigen loss during detoxification process [21].

3. The purity of the toxin increased after ultra filtration. So ultra-filtration is useful for the partial purification of tetanus toxin.
4. According to the preliminary curve the tetanus toxoid was found to be precipitated by the ammonium sulphate concentration between 12-24%. Thus this range can be used routinely for the purification of the tetanus toxoid once the consistency is demonstrated.

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