FACTORS AFFECTING THE STABILITY OF CLOSTRIDIAL TOXINS AND TOXOIDS AND THEIR EFFECT ON THE IMMUNE RESPONSE

BY

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SUMMARY

The prepared different clostridial toxins gave best permanent toxicity when stored at 4°C after adding stabilizer and adjusting the pH value to 8.0 (for C.perfringens type B, C.septicum and C.novyi type B toxins) and at 6.5 (for C.perfringens type D and C.tetani toxins) at the end of their fermentation period. Immune response of polyvalent clostridial vaccine prepared from these toxins gave higher antibody titers in immunized sheep using vaccine without pH controlling (about 6.5) and had stabilizer in their content than these with or without stabilizer and their pH value was adjusted to 8.0.

INTRODUCTION

Production of highly potent clostridial toxins is of a practical importance in the preparation of effective toxoids for purposes of immunization and preparation of vaccines.

Stability problems in relation to bacterial vaccines vary widely between different types of product. Generally tetanus toxoids show high stability of potency. Some components, in particular pertussis toxin, show inherent low stability and degrade on storage at refrigerator temperatures unless stabilized by a protein cross-linking agent. Multivalent vaccines may present especial stability problems because of the interaction of various components in the liquid state (Corbel, 1996).

The formulation of several antigens into a polyvalent vaccine may have an adverse effect on the stability or immunological response of one or more components. An example of this phenomenon was tetanus toxoid which generally show high stability of potency opposite to other clostridial antigens (Ellis, 1999). She also added that the formulation of multiple antigens with non-overlapping pH stability profiles into a single combination vaccine represents a real challenge, which can be met with one or two approaches: The first approach is to select an intermediate pH with respect to the stability of the different antigens. The second approach is to select the formulated pH that is compatible with the most labile antigen efforts then can be focused on maintaining the stability of labile antigen, by utilizing a number of proven procedures including chemical modification. The routine use of adding glycine as stabilizer to tetanus toxin before toxoiding may explain the high antibody

titre of tetanus toxoids rather than other clostridial antigens containing in the same vaccine.

These previous different statements were put in consideration in this work to focus on the problems that we have regarding the insufficient immune response to some clostridial antigens used for production of polyvalent clostridial vaccine. Also to evaluate the optimum conditions to keep different clostridial toxins efficient for a long time.

MATERIAL AND METHODS

I- Preparation of toxins:

Toxins of C.perfringens types B and D, C.septicum and C.novyi type B were prepared according to Gadalla et al. (1974). C.tetani toxin was prepared according to Rijks Instructions (1980). These prepared toxins were subjected to the following tests:

i- Effect of pH and temperature.

Each type of prepared toxins was divided into two equal parts, the first part was left at its maintained pH (about 6.5) and the other one was adjusted at pH 8. Each of these two parts was in turn divided into two parts, the first part was allowed to settle at room temperature (about 20-25 °C) and the other part was kept in the refrigerator at 4°C during all periods of testing their toxicity.

ii- Effect of stabilizers:

Three types of stabilizers were used:

1. Glycine was added as 0.5% of toxin.
2. Glycerol was added to a final concentration of 15%.
3. Casitone stabilizer which consist of casitone to make a concentration of 2%, sodium glutamate in 1%, sucrose added to make 4% concentration and gelatin in a concentration of 0.2%.

Each type of toxin was divided into four parts, one part was left as a control and stabilizers were added to the other three parts. Then all toxins were kept at 4°C during the time of the experiment.

*** Determination of toxins lethality (MLD):**

The minimum lethal dose (MLD) for all prepared toxins under test according to Gadalla et al. (1974), except that of C.tetani the MLD was determined according to Ahmed (1991).

II- Preparation of vaccines:

Toxins of different clostridial strains were prepared according to the methods as aforementioned. Each toxin was divided into 2 parts; the first one was left without stabilizer, to the second part casitone stabilizer was added at the same concentration used before. Then these two parts were divided into another two parts, part was left without adjustment of pH (about 6.5) and the other one was adjusted at pH 8. Detoxification of all toxins was done by adding 0.5% formalin, 1% alum was added as adjuvant to each detoxified toxin. A polyvalent vaccine containing all toxoids and whole culture of C.chauvoei were mixed in equal amounts, tetanus toxoid was added as 25 Lf/dose. Sterility and safety tests were carried out according to the regulation of British Veterinary Codex (1970).

*** Vaccination Schedules:**

Each vaccine was injected subcutaneously in a group of 4 Balady sheep, of 8-12 months old, in two doses (5 ml and 3 ml) with 3-4 weeks intervals.

*** Serum samples and antitoxin assays:**

All sheep were bled pre-vaccination and 2 weeks after the second dose. Serum samples were separated, pooled for each group and stored at -20°C until used. The antitoxic values expressed in (IU/ml) of all pooled serum samples were determined in Swiss white mice by serum neutralization test (SNT) as described by British Veterinary Pharmacopoeia (1985) for all clostridial antigens and according to Rijks Instructions (1989) for C.tetani.

RESULTS AND DISCUSSION

Although the clostridial vaccines have proven highly effective in controlling clostridial diseases, trials have been done to obtain a high degree of potency by improving the stability of clostridial toxins used in vaccine preparation.

Changes in pH and temperature presumably contribute either to the activation, preservation or destruction of clostridial toxins. Results in table (1) shows that all types of toxins stored at 4°C were more stable than their correspondents kept at room temperature and at different periods. From the same table, it is clear that these toxins react variably with pH. Some still stable with pH value 8 (alpha toxin of C.septicum, beta toxin of C.perfringens type B and alpha toxin of C.novyi type B). These results agree with that of Jansen (1961) and Pivnick et al. (1964) who found that beta toxin was kept better at 4°C when the pH was increased to 8. Other toxins like epsilon toxin of C.perfringens type D and tetanus toxin are more stable at obtained pH 6.5. This also agree to some extent to that obtained by Bernheimer (1944) who stated that final pH attained by cultures may hinder the formation of haemolysin or hastens its destruction. Regarding the periods that the toxins still active, epsilon and tetanus toxins were found to be the most active toxins for long periods (more than 6 months) followed by alpha toxin of C.novyi (5 months), for the other two toxins the periods range from 2-4 months. This variation may be due to the different level of toxicity for each toxin.

Addition of different stabilizers to the different clostridial toxins maintained their lethality for a period more than seven months with gradual decrease in their toxicity, in comparison to those without adding stabilizer (Table 2). Casitone stabilizer gave the best stability condition for beta and epsilon toxins of C.perfringens types B and D respectively, and alpha toxin of C.novyi type B. These results agree to that mentioned by Pivnick et al. (1964) who found that when a culture filtrate of C.perfringens type B mixed with casitone stabilizer, lyophilized and stored at 4°C, it gave constant values for lethality for at least one year. On the contrary, glycine stabilizer gave the best results with C.tetani toxin and alpha toxin of C.septicum. Glycerol stabilizer gave the lowest stability for all toxins.

Studying the immune response of the prepared polyvalent clostridial vaccine in which pH controlled and casitone stabilizer was added; Table (3) summarizes the results obtained, which indicates that the antibody titers for all antigens were higher in the vaccine which has been stabilized without pH adjustment (about 6.5) than those without stabilizer and pH 8.

These obtained results agree with that of El-Menisy (2000) who prepared a polyvalent clostridial vaccine in a lyophilized form using casitone stabilizer after toxoiding and found that it retained its antitoxic titre in sera of animals. Also, the vaccine without stabilizer and without pH controlling (pH about 6.5) gave higher antibody titre than that at pH 8 and without stabilizer for all antigens. These results may explain that obtained by Ellis (1999) who stated that the pH of the formulation may be altered to enhance binding with adjuvant. Antibody titre of tetanus was not affected by the addition of casitone stabilizer. This results agree with that of Emara (1998) who reported that when tetanus toxoid was lyophilized with casitone stabilizer, it did not retain its initial biological activity.

From this study it could be concluded that to keep clostridial toxins active for long period a suitable stabilizer for each toxin should be added and the suitable pH of the toxin should be at 8 for C.perfringens type B, C.septicum and C.novyi; and 6.5 for C.perfringens epsilon toxin and C.tetani. On the other hand, to prepare a potent and effective polyvalent clostridial vaccine stabilizers should be added before toxoiding and the pH was left as after the process of fermentation (about 6.5).

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Table (1) Effect of pH and temperature of storage on the potency of some clostridial toxins determined by MLD/ml

Days of storage	β (*C.perfringens*)				ε (*C.perfringens*)				*C.septicum*				*C.novyi* type B				*C.tetani* ×10³			
	pH 6.5		pH 8		pH 6.5		pH 8		pH 6.5		pH 8		pH 6.5		pH 8		pH 6.5		pH 8	
	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C
1st d	4000	4000	4000	4000	15000	15000	15000	15000	60	60	60	60	5000	5000	5000	5000	600	600	600	600
2nd d	0	0	4000	4000	10000	15000	10000	15000	10	40	20	60	5000	5000	5000	5000	600	600	600	600
3rd d			10	4000	ND	ND	ND	ND	0	30	10	60	5000	5000	5000	5000	600	600	600	600
4th d			0	ND	7000	12000	6000	10000		10	0	60	5000	5000	5000	5000	600	600	600	600
5th d				ND	ND	ND	ND	ND		10		60	5000	5000	5000	5000	600	600	600	600
6th d				ND	5000	8000	3000	7000		5		60	5000	5000	5000	5000	40	600	10	150
7th d				3000	3000	5000	2000	5000		0		60	5000	5000	5000	5000	ND	ND	ND	ND
14th d				3000	2500	5000	1500	4000				50	1000	2500	1500	2600	ND	ND	ND	ND
21st d				1000	2000	4500	1500	4000				40	800	1000	1000	1000	ND	ND	ND	ND
28th d				800	2000	4000	1000	3000				30	300	900	400	1000	6	50	0.8	8
60th d				500	1000	3000	1000	3000				10	50	600	100	800	0.6	10	0	3.8
90th d				200	800	2000	100	1500					0	300	0	500	0	8		2
120th d				50	300	1500	0	1200						50		100		6		1.5
150th d					0	1200		1000						0		50		5		1.3
180th d						1300		800										4		1.2

RT = Room Temperature (20-25°C). ND = Not Determined. d = day.

Table (2) Effect of stabilizers on the stability of some clostridial toxins

Period	β (*C.perfringens*)				ε (*C.perfringens*)				α (*C.septicum*)				α (*C.novyi*)				*C.tetani* × 10^4			
	WS	C	Gn	Gl	WS	C	Gn	Gl	WS	C	Gn	Gl	WS	C	Gn	Gl	WS	C	Gn	Gl
0	3000	3000	3000	3000	8000	8000	8000	8000	80	80	80	80	1000	1000	1000	1000	80	80	80	80
1st w	400	1000	400	400	3000	5000	3000	5000	80	80	80	80	400	700	500	400	18	18	18	18
2nd w	300	800	350	400	2000	5000	3000	3000	70	70	80	65	300	600	400	300	3.8	8	12	3.6
3rd w	150	700	300	400	2000	4000	2500	2000	60	70	70	60	200	500	300	200	3.6	6	6	3.2
4th w	150	700	250	400	1500	3000	2000	2000	50	60	70	55	200	400	200	200	2	5	6	1.6
8nd w	100	400	250	300	1000	2500	1500	1500	40	60	65	45	150	300	150	150	1.6	4	6	1.2
12rd w	80	250	200	300	800	1500	2000	1500	30	50	60	30	100	200	70	70	1.2	3.2	5	0.4
16th w	60	250	150	200	700	1800	1200	1000	25	40	45	30	30	150	50	50	0.9	2	3	0.2
20th w	30	200	100	150	700	1800	1000	1000	20	30	30	25	35	100	40	30	0.8	1	2	0.009
24th w	15	100	50	70	500	1500	800	800	20	20	30	20	30	80	30	15	0.6	0.8	1.6	0.001
28th w	-ve	100	20	20	500	1000	700	600	10	15	20	10	20	50	20	10				

WS = toxin without stabilizer. C = toxin with casitone. W = weeks.
Gn = toxin with glycine. Gl = toxin with glycerol.

Table (3) Antibody titers in sera of sheep vaccinated with different polyvalent clostridial vaccines

Type of antitoxin	Mean antibody titer (IU/ml)			
	Vaccine without pH adjustment	Vaccine without pH adjustment + stabilizer	Vaccine with pH adjusted at 8	Vaccine with adjusted pH at 8 + stabilizer
β antitoxin of C.perfringens type B	45	60	11	30
ε antitoxin of C.perfringens type D	35	45	18	30
α Antitoxin of C.novyi type B	30	40	9	14
Antitoxin of C.tetani	5.12	5.12	1.28	1.28
α Antitoxin of C.septicum	2	10	0	7

- Pre-vaccination titer in animals was zero.

الملخص العربى

العوامل المؤثرة على ثبات سموم وتوكسيدات ميكروبات الكلوستريديا وتأثيرها على الإستجابة المناعية

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فى هذه الدراسة وجد أن إضافة المثبتات المختلفة مع ضبط التركيز الأيونى الهيدروجينى عند تركيز ٨ (لسموم ميكروبات الكلوستريديم بيرفرنجنز نوع "ب" والكلوستريديم سبتكم والكلوستريديم نوفياى نوع "ب") وعند تركيز ٦,٥ (لسموم الكلوستريديم برفرنجنز نوع "د" والكلوستريديم تيتناى) وذلك فى نهاية مدة التخمر يجعلها تحتفظ بدرجة سميتها لفترة طويلة عن مثيلاتها التى لم تضاف إليها المثبتات ولم تضبط لها درجة الأس الهيدروجينى وذلك عند حفظها فى الثلاجة. وقد وجد أن الإستجابة المناعية للأغنام المحصنة باللقاح الجامع المحضر من تلك السموم ولم يضبط له الأس الهيدروجينى والمضاف إليها المثبت أعلى من الإستجابة المناعية الناتجة من اللقاح الذى لم يضاف إليه المثبت وكذلك الذى تم ضبط التركيز الأيونى الهيدروجينى إلى ٨ أضيف أو لم يضاف إليه المثبت.