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Preparation and Potency Testing of Long Acting Tetanus Toxoid Microcapsules

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ABSTRACT: In recent years, attempts have been made to develop microcapsule-type tetanus toxoid with the aim of prolonging the potency of tetanus toxoid to reduce the frequency of immunization. A non-toxic, biocompatible and biodegradable substance like egg yolk lecithin was employed in this study to prepare long acting tetanus toxoid microcapsules which may help alleviate some of the problems associated with conventional vaccine. Long acting tetanus toxoid microcapsules were prepared by the modified interfacial deposition technique using egg yolk lecithin as a wall material, while carboxymethylchitin was used to strengthen the wall by electrostatic binding. The microcapsules were separated by centrifugal technique and were divided according to their particle size into three groups, namely A, B and C. To compare the potencies of the above "long acting" tetanus toxoid microcapsules with the conventional vaccine, eight groups of mice, each consisting of 140, were immunized with 1-8 formula samples: PBS pH 7.4 (as a control), tetanus toxoid, tetanus toxoid microcapsules A, tetanus toxoid microcapsules B, tetanus toxoid microcapsules C, mixture of tetanus toxoid and tetanus toxoid microcapsules A, mixture of tetanus toxoid and tetanus toxoid microcapsules B, mixture of tetanus toxoid and tetanus toxoid microcapsules C, respectively. Ten of each group of mice were challenged with 200 LD_{50} toxin at days 0, 3, 7, 15, 30, 45, 60, 75, 90, 105, 120, 140, 160 and 180 after immunization. The quantity of survived mice on the fifth day after being challenged was observed. Tetanus toxoid could completely protect the mice only from days 30 to 75 but not later. The protection of tetanus toxoid microcapsules started between days 30-120 and lasted longer than the unencapsulated toxoid. Tetanus toxoid microcapsules B gave more protection than A and C. Furthermore, the mixture of tetanus toxoid and tetanus toxoid microcapsules B gave the best immune response and longest duration. The protective level was found to persist from days 15-180.

KEY WORDS: microcapsules, tetanus toxoid.

INTRODUCTION

Tetanus is a global disease that accounts for approximately 50% of neonatal deaths and 25% of infant deaths. Current estimates of world wide mortality are 800,000 deaths per year from neonatal tetanus and 120,000 to 300,000 deaths per year from non-neonatal tetanus. This high incidence is additionally tragic because the disease is largely preventable by appropriate immunization. The WHO Expanded Programme on Immunization or EPI (1) has been working toward greater vaccine coverage particularly in developing countries, in the hope that tetanus will someday become a rare disease.

The outstanding property of the tetanus baccillus is its capacity to produce a powerful, specific exotoxin. The toxin is a simple protein, a polypeptide chain of approximately 150,000 Da (2). The molecule can be cleaved by clostridial proteases to form two chains linked by a disulfide bond. In purified form 1 mg may contain enough toxin to kill 35 million mice. Human beings are susceptible to this toxin. It has a peculiar affinity for nervous tissue, including the

peripheral nerves, and especially for the motor nerve centers in the central nervous system. When the poison reaches these centers tetanic convulsion of the muscles follows.

The crude toxin is detoxified before purification with a dilute solution of formaldehyde for a period of days or weeks, and is usually purified by alcohol or salt fractionation, during which time the product may be tested for toxicity in animals. The product is labeled as tetanus toxoid only when it no longer exhibits any signs of tetanus toxicity. Adsorbed tetanus toxoid is the toxoid which has been adsorbed on to aluminium hydroxide, aluminium phosphate or potassium alum. The antigen content of toxoid is expressed in flocculating unit. Each 0.5 ml of available adsorbed tetanus toxoid contains 5 or 6 floccalating units of tetanus toxoid.

Tetanus toxoid and adsorbed tetanus toxoid promote active immunity to tetanus by inducing production of specific antitoxin. A single intramuscular or subcutaneous injection dose of either type of toxoid does not provide protection against the disease. The intramuscular route is preferred for adsorbed tetanus toxoid. The usual dose of the tetanus toxoid products is 0.5 ml. For primary immunization of infants and young children, tetanus toxoid is administered in a series of 3 doses. The second dose is given at 4-8 weeks after the first dose and the third dose given at 6-12 months after the second dose.

The problems of incomplete immunizing programme of tetanus toxoid will cause more incidence and deaths. The objective of this work is thus to develop a new, more effective, long acting tetanus toxoid microcapsules to reduce the frequency of immunization by using only a single dose instead of triple dose given at 2, 4 and 6 months. Lecithin and carboxymethylchitin walled tetanus toxoid microcapsules were developed by interfacial deposition technique in the preparation of long acting tetanus toxoid microcapsules.

MATERIALS AND METHOD

Materials

Adsorbed tetanus toxoid (TT), and tetanus toxin were received from the Government Pharmaceutical Organization, Bangkok, Thailand. Carboxymethylchitin was prepared by interaction of sodium monochloroacetate and chitin as described by Kato et al (3). Purified egg yolk lecithin was received from Asahi Kasei Chemical Industries Ltd, Tokyo, Japan. All other chemicals were of reagent grade.

Methods

Preparation of lecithin and carboxymethylchitin walled tetanus toxoid microcapsules: Tetanus toxoid microcapsules were prepared by modified interfacial deposition technique (4,5). Purified egg yolk lecithin was dissolved in dichloromethane at 50 mg/ml. Carboxymethylchitin was dissolved in a phosphate buffer solution pH 7.4 at a concentration of 0.2 percent w/v. An equal volume of the lecithin solution was added to 10 ml of adsorbed tetanus toxoid, and the mixture was vigorously agitated by vortex mixer for 30 seconds to give a w/o emulsion. The emulsion obtained was quickly added with stirring to 100 ml of carboxymethylchitin solution to yield a w/o/w emulsion. After 10 minutes stirring, another 100 ml of the carboxymethylchitin solution was added to the complex emulsion under stirring and the stirring was further continued for 24 hours until the dichloromethane was completely evaporated. Lecithin and carboxymethylchitin walled tetanus toxoid microcapsules were centrifuged at 2000 rpm for 15 minutes. The microcapsules were washed three times with phosphate buffer solution pH 7.4. The microcapsules were subsequently weighed and redispersed in the same buffer. This preparation was referred to as tetanus toxoid microcapsules A (TTMA). The supernatant was continued to centrifuge at 5,000 rpm for 20 minutes and 12,000 rpm for 30 minutes. After each centrifugation, the microcapsules were washed and collected in the same way, yielding tetanus toxoid microcapsules B (TTMB) and tetanus toxoid microcapsules C (TTMC), respectively. The volume of the tetanus toxoid microcapsules was adjusted to the same volume as the original tetanus toxoid. So the concentration of microcapsules was 16.5% w/v. The mixtures of TT and TTMA, TT and TTMB, TT and TTMC were subsequently prepared, all at the ratio 1:1.

Scanning electron microscopy (SEM). A scanning electron microscope (JSM-T220A, Jeol Co. Ltd, Tokyo, Japan) was used to observe the surface morphology of the microcapsules.

Particle size analysis. A coulter counter (Hyad/Royco Model 300) was used to determine the diameter of TTMA, TTMB and TTMC.

Determination of $LD_{50/ml}$. Tetanus toxin was diluted to concentration of 10^{-1}, 10^{-2},10^{-3}, 10^{-4}, 10^{-5},10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} times with phosphate buffer solution pH 7.4. 0.5 ml

of each dilution was injected subcutaneously into 10 mice (17-20 g body weight/mice), and then observed for 5 days. Accumulated died value D, accumulated survived value S, accumulated mortality ratio and $LD_{50/ml}$ were calculated.

Potency testing (6). The potencies of adsorbed tetanus toxoid (TT), tetanus toxoid microcapsules A (TTMA), tetanus toxoid microcapsules B (TTMB), tetanus toxoid microcapsules C (TTMC), and the mixtures of TT and TTMA, TT and TTMB, TT and TTMC in mice were determined during 180 days after immunization and compared with the control group of mice which received only PBS pH 7.4. Ten mice of each group of the immunized mice were challenged with 200 $LD_{50/ml}$ of tetanus toxin at days 0, 3, 7, 14, 30, 45, 60, 75, 90, 105, 120, 140, 160 and 180. The quantity of survived mice on the fifth day was recorded. The results of each group were compared by an appropriate statistical method.

Antibody determination (7-9)

Blood was withdrawn from each group of mice immunized with TT, TTMA, TTMB, TTMC, the mixtures of TT and TTMA, TT and TTMB, TT and TTMC by heart puncture technique. The presence of antibodies was determined by a passive hemagglutination technique (PHA) with indicator sheep erythrocytes optimally conjugated with tetanus toxoid. Two fold dilutions of heart inactivated serum in saline with 1% normal rabbit serum (heart inactivated and adsorbed with sheep erythrocytes) were made in microtiter plates. A 50 µl amount of suspension of indicator erythrocytes was mixed with 50 µl of serum dilution. The PHA was read after 16 hours and the reciprocal value of the greatest dilution still giving agglutination was taken as the titer.

RESULTS AND DISCUSSION

Tetanus toxoid microcapsules were prepared by interfacial deposition technique. The lecithin will coat around tetanus toxoid droplets and carboxymethylchitin will interact with the hydrophobic group of the lecithin molecules oriented at the oil-water interface to form a stable adsorbed layer on the surface of vesicles. The spherical shape of the vesicles was obtained by controlling the speed of stirring during the process of preparation. On separation of various vesicle sizes of tetanus toxoid microcapsules, large microcapsules TTMA and traces of lecithin were obtained at the lower speeds of centrifugation (2000 rpm). The yield was 41.2 percent and the mean diameter was 5.07 µm. The medium and the small microcapsules, TTMB and TTMC, were obtained at the respective speed of 5,000 and 12,000 rpm. The yields were 49.7 percent and 10 percent, with the mean diameter of 3.77 µm and 2.97 µm, respectively. Figure 1 shows aggregation of the tetanus toxoid microcapsules in which the individual microcapsules ranged from 0.2-0.5 µm. Scanning electron micrograph also shows the spherical and smoothly walled microcapsules. The cumulative percent undersize distribution curves are also illustrated in Figure 2 for the three microcapsules.

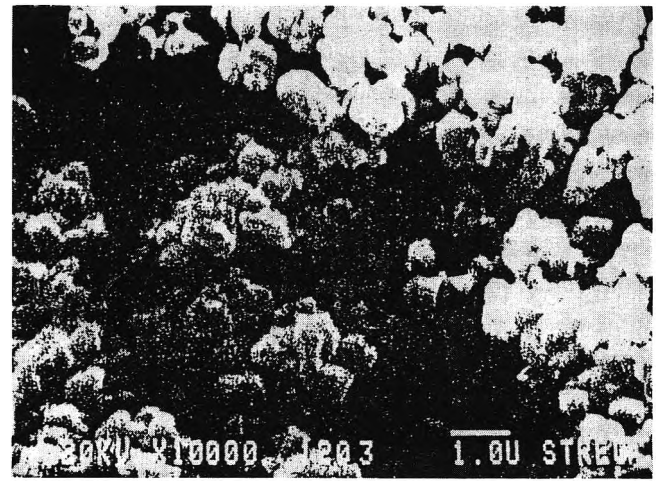

Figure 1 Scanning electron micrograph of tetanus toxoid microcapsules

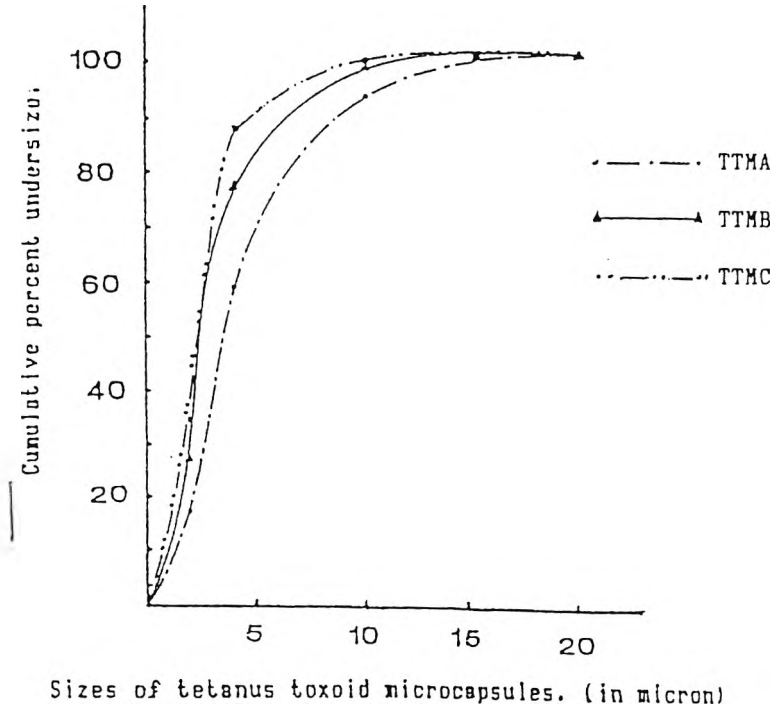

Figure 2 The cumulative percent undersize distribution curves of tetanus toxoid microcapsules.

Determination of $LD_{50/ml}$. The concentration of the toxin that produced 50% mortality in mice at the fifth day after being injected subcutaneously with 0.5 ml toxin was observed. There were eight-tenth mice died at the dilution of 10^{-5} tetanus toxin, and all of them survived at the dilution of 10^{-6}. Thus, the dilution that produced 50% or five-tenth mortality in mice should be the dilution between 10^{-5} and 10^{-6}. So the proportionate distance was calculated. Therefore, the appropriate dilution for 50% mortality in mice of tetanus toxin or $LD_{50/ml}$ tetanus toxin was $10^{-5.375}$. If the desired concentration was 200 $LD_{50/ml}$, the toxin could be diluted at the ratio of 1:1185.69 ml when the calculated $LD_{50/ml}$ of toxin was $10^{-5.375}$.

Potency testing: Table 1 shows that at days 0-3 all of the mice challenged with 200 $LD_{50/ml}$ tetanus toxin in every group of samples died. Immunization with TT and mixtures of TT and tetanus toxoid microcapsules showed the protective effect on day 7. They could protect 2-3 mice whereas the TTMA, TTMB and TTMC had no effect. At day 45 TT could completely protect all 10 mice. However, the mixtures of TT and the microcapsules A, B and C were able to completely protect all 10 mice at earlier time, i.e. day 30. The adsorbed tetanus toxoid showed the shortest onset while the tetanus toxoid microcapsules showed longer onset. TTMC could completely protect all 10 mice at day 45 while TTMA and TTMB could completely protect 10 mice at day 60. The adsorbed tetanus toxoid could protect mice until only day 90, after that all 10 mice died. TTMB could completely protect all 10 mice until day 180 and was found to give more protection than TTMA and TTMC. The mixture of TT and TTMB gave the shortest onset and longest duration. The protective level persisted during days 15-180.

The adsorbed tetanus toxoid had the shortest onset and duration while the tetanus toxoid microcapsules had the longer onset and duration because they could not immediately induce the immune response. In microcapsules, the toxoid was slowly released through the polymeric membrane for a longer period, resulted in a prolonged immunizing activity of the toxoid.

Table 2 shows the mean titers after immunization with TT, TTMA, TTMB, TTMC, TT+TTMA, TT+TTMB, TT+TTMC, and PBS pH 7.4. At day 3 the mean titers after immunization with adsorbed tetanus toxoid TT, TTMC and the mixtures of TT and TTMA, TTMB and TTMC were found at the low concentration. They started from 0.07-0.15 unit/ml but they could not protect the mice. All the mice in Table 1 at day 3 died. At day 7 the mean titer in mice immunized with TTMC was 0.45 unit/ml. But it still could not protect the mice since all 10 mice in Table I did not survive. The mean titers in mice immunized with TT,

TT+TTMA, TT+TTMB, TT+TTMC were 0.63-0.85 unit/ml while in Table 1 the number of survived mice which were immunized with tetanus toxoid was 2-3. At day 45 the number of survived mice which were immunized with TT was 10 and the mean titer was 2.10 unit/ml and remained constant until day 75. As a result, the mean titer which could protect mice appeared to be 2.0 unit/ml. The mean titer in mice immunized with TTMC was 2.0 unit/ml at day 30 and it could protect 9 mice. However, the mean titers in mice immunized with TT+TTMA, TT+TTMB, TT+TTMC were 2.6-2.8 unit/ml and they could completely protect all 10 mice.

In order to save all mice from tetanus toxin, the mean titers should be higher than 2.0 unit/ml. The mixtures of TT and the tetanus toxoid microcapsules will increase the titer in mice. After the highest titer level, the value decreased. However, all of the mice were still safe even though the mean titers in mice immunized with the tetanus toxoid microcapsules could be as low as 1.1 unit/ml. Most of the mice died when the titer levels were lower than 0.5 unit/ml. At the earlier period the protective titers were higher than that of the final period. In consideration, immunoglobulin M (IgM) is the main immunoglobulin produced early in the primary response, followed by immunoglobulin G (IgG) and continues to rise for a long period, after this it declines and drops to very low level. IgM declines and only IgG remains (10). IgG is more specific in neutralizing tetanus toxin than IgM. Although the titer levels were lower than 2.0 unit/ml (e.g. 1.1 unit/ml), it still had the protective effect. When the titers decreased below 0.5 unit/ml, they could not save the mice any further.

Table 1 Number of survived mice which were immunized with tetanus toxoid (TT), tetanus toxoid microcapsules (TTMA, TTMB, TTMC), and the mixtures of tetanus toxoid and tetanus toxoid microcapsules 1:1

Preparation / Day	TT	TTMA	TTMB	TTMC	TT+ TTMA	TT+ TTMB	TT+ TTMC	PBS pH7.4
0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
7	3	0	0	0	2	3	2	0
15	5	0	2	3	6	6	5	0
30	9	3	7	9	10	10	10	0
45	10	5	8	10	10	10	10	0
60	8	10	10	10	10	10	10	0
75	7	10	10	10	10	10	10	0
90	3	10	9	7	10	10	7	0
105	0	10	10	3	10	10	3	0
120	0	10	10	1	10	10	1	0
140	0	8	10	0	8	9	0	0
160	0	7	10	0	7	10	0	0
180	0	5	10	0	4	10	0	0

Table 2 The mean titers in mice which were immunized with tetanus toxoid (TT), tetanus toxoid microcapsules (TTMA, TTMB, TTMC), and the mixtures of tetanus toxoid and tetanus toxoid microcapsules 1:1.

Preparation / Day	titer (unit/ml)							
	TT	TTMA	TTMB	TTMC	TT+ TTMA	TT+ TTMB	TT+ TTMC	PBS pH7.4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.13	0.00	0.00	0.07	0.15	0.12	0.15	0.00
7	0.63	0.00	0.25	0.45	0.80	0.85	0.80	0.00
15	1.00	0.06	0.65	0.75	1.60	1.60	1.60	0.00
30	1.40	0.10	1.80	2.00	2.60	2.60	2.80	0.00
45	2.10	0.45	2.60	2.80	3.20	3.40	3.40	0.00
60	2.00	0.75	3.20	3.40	3.20	3.40	2.50	0.00
75	2.00	1.80	3.40	1.00	3.20	2.80	1.50	0.00
90	0.55	2.00	3.40	1.00	2.20	2.50	0.85	0.00
105	0.45	1.70	2.40	0.60	1.70	2.20	0.50	0.00
120	0.06	1.20	2.10	0.25	1.10	1.90	0.40	0.00
140	0.00	0.95	1.85	0.45	0.95	1.40	0.40	0.00
160	0.00	0.85	1.50	0.40	0.85	1.10	0.20	0.00
180	0.00	0.78	1.20	0.40	0.80	1.20	0.20	0.00

CONCLUSION

The adsorbed tetanus toxoid (TT) had the shortest onset and duration. It could completely protect the mice only from days 30 to 75 but not later. Tetanus toxoid microcapsules had the longer onset and duration. The protection of tetanus toxoid microcapsules started from days 30 to 120 and lasted longer for at least 180 days. The mixture of TT and TTMB gave the shortest onset and longest duration. It could potentiate the immune response of tetanus toxoid microcapsules leading to high blood level of antibodies. The mixture of TT and TTMB appeared to give the best immune response and longest duration. The protective level persisted from days 15 to 180.

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REFERENCES

1. World Health Organization. WHO manual for the production and control of vaccines tetanus toxoid. WHO technical report series 800. Requirements for tetanus toxoid general considerations, Geneva, 1990, pp.109-126.
2. B. Helting, S.Parschats and H. Engehardt. Structure of tetanus toxin demonstration and separation of a specific enzyme converting intracellular toxin to the extracellular form. *J. Biol. Chem.* 254:10728-10733 (1979).
3. A. Kato, M. Arakawa and T.Kondo. Preparation and stability of liposome-type artificial red blood cells stabilized with carboxymethylchitin. *J. Microencapsulation.* 1(2): 105-112 (1984).
4. A. Kato and T.Kondo. A study of liposome-type artificial red blood cells stabilized with carboxymethylchitin. *Polym. Sci. Tech.* 35:299-310 (1987).
5. A. Izawa and T. Kondo. Disintegration by surfactants of egg yolk phosphatidylcholine vesicles stabilized with

carboxymethylchitin. *Biochem. Biophys. Acta.* 885: 243-249 (1986).

6. R. Mittal, T. Jaiswal and K. Gupta. Study on haemorrhagic septicemia of oil adjuvant and multiple emulsion adjuvant vaccines II: immunity trial in mice, rabbits and calves. *Indian Vet. J.* 56:449-454 (1979).
7. D.P. Stites. Clinical Laboratory Methods for Detection of Antigens and Antibodies. In D.P. Stites, I.D. Stobo, H.H. Fundenberg and J.V. Wells (eds.), *Basic and Clinical Immunology*, 4th ed., Lange Medical Publication, Maruzen Asia, 1982, pp.325-365.
8. E.S. Glolub. *Immunology*. A Synthesis Sinauer Associate, Inc. Publishers, Sunderland, Massachusetts, 1987, pp. 120-135.
9. W.T. Herbert. Passive Haemagglutination. In D.M. Weir (ed.), *Handbook of Experimental Immunology*, Blackwell Scienctific Publication, Oxford, 1967, pp. 720-724.
10. R.M. Hyde. *Immunology*, The National Medical Series for Independent Study, 2nd ed., Harwal Publishing, 1992, pp.80.