Chapter III

Results and Discussion

1) Preparation of Lecithin and Carboxymethylchitin Walled Tetanus Toxoid Microcapsules.

Tetanus toxoid microcapsuleswere prepared by interfacial polymerization technique. The microcapsules wall obtained by adding 0.2% lecithin in dichloromethane in equal volume to tetanus toxoid. During stired this mixture,w/o emulsion was obtained. As this, the lecithin would coated around tetanus toxoid droplets. To stabilize the lecithin wall, carboxymethylchitin (CMC) in phosphate buffer solution pH 7.4 (PBS pH 7.4)was added. At pH 7.4 lecithin molecule had a net positive charge while CMC molecule in the outer aqueous phase would interact electrostatically woth the hydrophobic group of the lecithin molecules oriented at the oil-water interface to form a stable adsorbed layer on the surface of oil droplets as in figure 14.

The spherical shape of vesicle were obtained by controlling the speed of stirring. In processing of preparation,adding another portion of CMC in PBS pH 7.4 help to strengthen the microcapsules wall. In according to the solubility of lecithin that is soluble in dichloromethane , an organic solvent, but insoluble in water. Therefore , evapolation of dichloromethane from the system would strengthen the microcapsules walls.

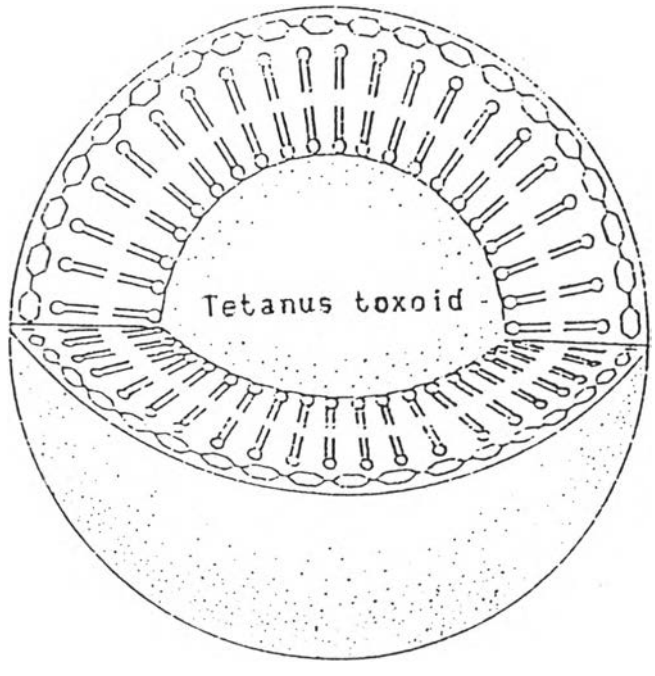

Figure 14 Structure of lecithin and carboxymethyl chitin walled tetanus toxoid microcapsules by interfacial polymerization technique.

2) Separation of Various Vesicle Size Preparation of Tetanus Toxoid Microcapsules by Centrifugation Method.

As in the experiment, at the lower speeds of centrifugation, 2,000 rpm, larger microcapsules and traces of lecithin were obtained,and the smaller microcapsules were remained in the supernatant.Tetanus toxoid microcapsules were obtained at a rate that was related to their size and density. Large microcapsules and traces of lecithin could easily be pelleted at fairy low gravitational forces in a conventional centrifuge.

If all of it contained traces of lecithin, ones can discarded this portion but large microcapsules that might prolong the action of tetanus toxoid were also present, therefore collecting the portion for quality testing of tetanus toxoid had been done.

At the higher speed, 5,000 rpm ,the medium size microcapsules might be obtained. And at the highest speed, 12,000 rpm,the smallest microcapsules were collected. Most of the precipitates were collected from 2,000 and 5,000 rpm centrifugation(41.2 % and 49.7 % of total precipitates) and a small amount(less than 10 % of total precipitates) of the precipitate was collected from 12,000 rpm centrifugation.

In redispersing of the microcapsules, only iso-osmotic pressure medium was used to stabilized the microcapsules. So phosphate buffer saline solution pH 7.4 (PBS pH 7.4),an iso-osmotic pressure solution was choosen. The volume of tetanus toxoid microcapsules were adjusted to the same volume as the original tetanus toxoid by adding PBS. pH 7.4 . As the total precipitation was 66.0 grams , the original tetanus toxoid volume was 400 ml. So the concentration of the microcapsules was 16.5 % w/v. The portions of precipitation was redispersed into three preparations as

TTMA, TTMB, and TTMC respectively.

3) Testing the quality of Tetanus Toxoid Microcapsules

3.1) Physical Testing

a) Particle Size Analysis

The size distribution of each preparation were difference. The cumulative percentage undersize distribution was shown in table 5 . Mean diameter range of TTMA that was separated from the centrifugation at 2,000 rpm 15 minutes was 5.07 micrometers. Mode or the diameter range that had the most frequency of vesicle was between 2.1-5.0 micrometers in 40 % frequency. Median, the diameter range at the 50 % of cumulative percentage undersize was 4.23 micrometers.

Table 6 shows the cumulative percentage undersize distribution of preparation ,TTMB , that was separated from the supernatant after the first separation, by centrifugation at 5,000 rpm 20 minutes. Mean diameters was 3.77micrometers ; mode was between 2.1-5.0 micrometers in 50.49 %frequency; and median was 3.25 micrometers.

Table 7 shows the cumulative percentage undersize distribution of preparation TTMC that was separated from the supernatant after the second separation at 12,000 rpm 30 minutes. Arithmetic mean was 2.94 micrometers; mode was between 2.1-5.0 micrometers in 53.32 % frequency; and median was 2.89 micrometers.

In comparison of the size distribution of these three preparations TTMA, TTMB, TTMC (Figure 15), the mode were in the same range ;2.1-5.0 micrometers but the percent frequency in these ranges of mode were difference ; 40.53 % in TTMA, 50.49 % in TTMB , and 53.32 % in TTMC.

Table 5 The cumulative percentage undersize distribution of "TTMA" that obtained from 2,000 rpm centrifugation.

Size range (micrometers)	% frequency	% cumulative
1.1- 2.0	18.73	18.73
2.1- 5.0	40.53	59.26
5.1-10.0	33.59	92.85
10.1-15.0	6.78	99.63
15.1-20.0	0.32	99.95
Arithmetic mean = 5.07 micrometers		
Mode = 2.1-5.0 micrometers		
Median = 4.23 micrometers		

Table 6 The cumulative percentage under size distribution of "TTMB" that obtained from 5,000 rpm centrifugation.

Size range (micrometers)	% frequency	% cumulative
1.1- 2.0	28.32	28.32
2.1- 5.0	50.49	78.81
5.1-10.0	19.36	98.17
10.1-15.0	1.74	99.91
15.1-20.0	0.10	100.01

Arithmetic mean	= 3.77	micrometers
Mode	= 2.1-5.0	micrometers
Median	= 3.25	micrometers

Table 7 The cumulative percentage undersize distribution of "TTMC"that obtained from 12,000 rpm centrifugation.

Size range (micrometers)	% frequency	% cumulative
1.1- 2.0	33.84	33.84
2.1- 5.0	53.32	87.16
5.1-10.0	12.03	99.19
10.1-15.0	0.74	99.93
15.1-20.0	0.06	99.99

Arithmetic mean	= 2.94	micrometers
Mode	= 2.1-5.0	micrometers
Median	= 2.89	micrometers

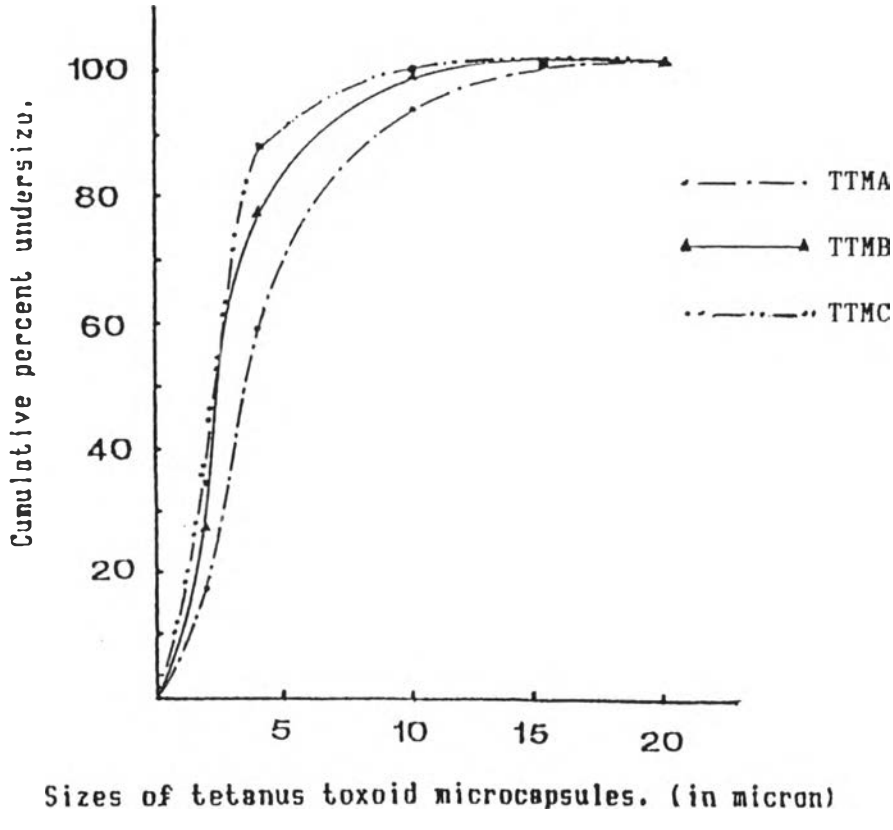

Figure 15 The cumulative percent under size distribution curves of tetanus toxoid microcapsules.

Median were 4.33 micrometers in TTMA , 3.25 micrometers in TTMB and 2.89 micrometers in TTMC. Arithmetic mean were 5.07 micrometers in TTMA , 3.77 micrometers in TTMB and 2.94 micrometers in TTMC.

According to the results, they were correlated to the theory that the larger vesicle size , the lower speed needed for separation. At 2,000 rpm, the low speed, mean diameter of vesicle were large; 5.07 micrometers but when the speed of centrifugation were higher, 5,000 rpm and 12,000 rpm,the mean diameter of vesicle were decreased;3.77 and 2.94 micrometers.

Not only arithmetic mean , the median were also confirmed these results; 4.23 micrometers in TTMA; 3.25 micrometers in TTMB and 2.89 micrometers in TTMC. In addition, although all speeds produced the same range in mode,2.1-5.0 micrometers,but the percent frequency in each speed were difference. At the highest speed, 12,000 rpm,the highest percent frequency was obtained. The lowest speed , 2,000 rpm ,the lowest percent frequency (40.53 %) was also obtained.

The reason was that at low speed most of the larger microcapsules were pelled as the gradient, While smaller microcapsules at the higher speed the needed more gravitational force for separation. Hence most of the smaller microcapsules were obtained at the higher speed.

Although the centrifugation technique could separate the microcapsules according to the desired vesicle sizes , but this method have limitations in the operation. First, it is useful for pilot scale operation , not suitable for manufacturing because the volume of sample were limited by such instrument in the centrifugation method. The second is that the small microcapsules tend to require high speeds and long spinning time in order to achieve effective separation.

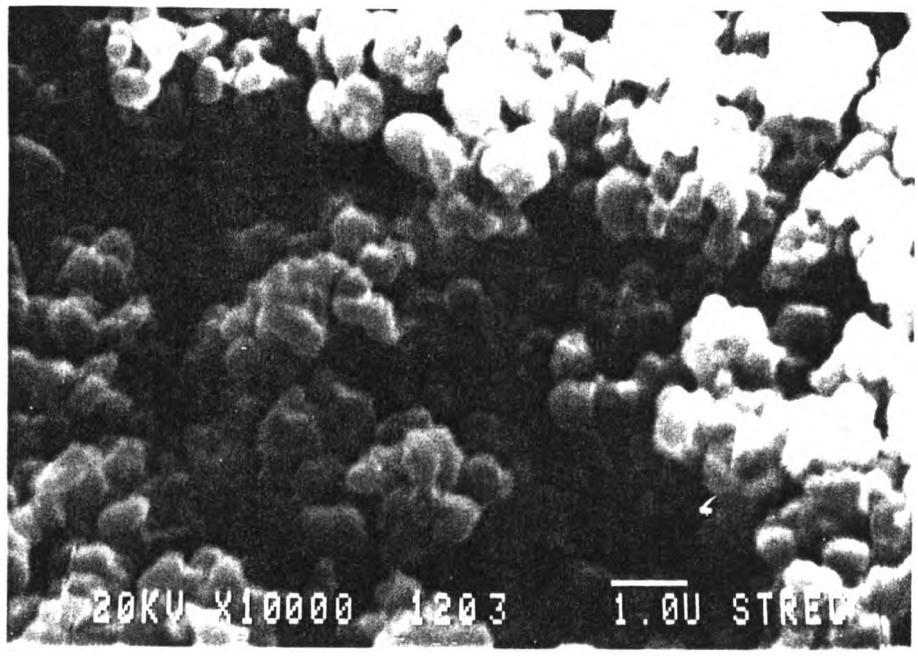

Figure 16 Scanning electron micrograph of tetanus toxoid microcapsules.

b) Scanning Electron Microscopy

Scanning electron microscopy showed spherical and smooth walls particles (Figure 16) of tetanus toxoid microcapsules. As the lecithin molecules , amphiphilic phospholipid , with a polar headgroup and two long fatty acid chains (Figure 17) represent only one type of several possible assemblies form , bilayer vesicle lecithin.

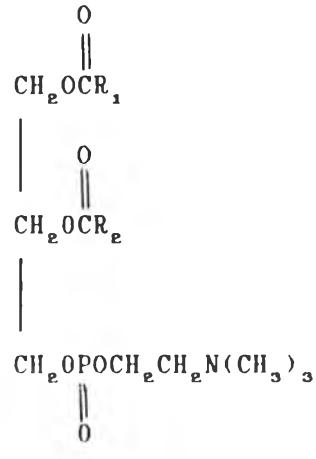

Figure 17 Structure of lecithin.

The shape of the amphiphilic molecule showed types of lipid assemblies under the right conditions. Two chain lipids resemble truncated cone or cylinders to form bilayers. A flat bilayer would be the most stable form ; due to unfavorable edge effects , however , such a bilayer would close upon itself , forming vesicles. According to these a tetanus toxoid droplet was enclosed within a bimolecular layer of lecithin molecules , to produce spherical vesicle which was further covered by an adsorbed layer of CMC molecules.

3.2) Animal Testing

a) $LD_{50/ml}$ of Tetanus Toxin

Table 8 showed number of mortal mice after injection with tetanus toxin in dilutions of 10^{-2},10^{-3},10^{-4},10^{-5},10^{-6},10^{-7}, and 10^{-8}. And the calculations of $LD_{50/ml}$ were also shown in table 8. $LD_{50/ml}$ was used to determine the concentration of the toxin that produced 50 % mortality mice in the fifth days after be injected with the toxin 0.5 ml subcutaneously.

As in table 8,there were eight-tenth mice died in the dilution of 10^{-5} tetanus toxin,and all of them were survived in the dilution of 10^{-6}. Thus, the dilution that produced 50 % or five-tenth mortality mice should be the dilution between 10^{-5} and 10^{-6}. So proportionate distance, 0.375, was calculated. The proportionate distance indicated that the desired concentration was more dilute $10^{0.375}$ times than 10^{-5} dilution. Therefore the appropiate dilution for 50 % mortality mice of tetanus toxin or $LD_{50/ml}$ tetanus toxin was $10^{-5.375}$. If desired concentration was 200 $LD_{50/ml}$,the toxin could be diluted 1:1185.69 ml when the $LD_{50/ml}$ of the toxin was $10^{-5.375}$ as the calculation.

Table 8 No.of survived mice and determination of $LD_{50/ml}$ of tetanus toxin.

Dilution	Mortality rate	Died	Survived	Accumulated Value			
				Died (D)	Survived (S)	Mortality ratio	Percent [D/(D+S)x100]
10^{-2}	10/10	10	0	38	0	38/40	100
10^{-3}	10/10	10	0	28	0	28/40	100
10^{-4}	10/10	10	0	18	0	18/40	100
10^{-5}	8/10	8	2	8	2	8/40	80
10^{-6}	0/10	0	10	0	12	0/10	0
10^{-7}	0/10	0	10	0	22	0/20	0
10^{-8}	0/10	0	10	0	32	0/30	0

$\therefore LD_{50/ml} = 10^{-5.375}$

Calculation of $LD_{50/ml}$

$$\text{Proportionate distance} = \frac{\text{mortality above 50\% - 50}}{\text{mortality above 50\% - mortality below 50\%}}$$

$$= \frac{80 - 50}{80 - 0}$$

$$= 0.375$$

$-\log LD_{50/ml}$ = -log dilution above 50% mortality + proportionated distance.

$$= -\log 10^{-5} + 0.375$$

$$= 5.375$$

Hence , $LD_{50/ml} = 10^{-5.375}$

If desired concentration was ;

1 $LD_{50/ml}$	tetanus toxin	1 ml	should be diluted into	$10^{5.375}$	ml.
200	"	200	"	$10^{5.375}$	"
200	"	1	"	$\frac{10^{5.375}}{200}$	"
				= 1185.69	"

3.2) Potency Testing

To determine and compare the potency of tetanus toxoid preparations in mice during 180 days after immunization,the mice were immunized with tetanus toxoid preparations and challenged with 200 $LD_{50/ml}$ tetanus toxin at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 105,120, 140, 160, and 180. The quantity of survived mice were recorded at the fifth days.

Table 9 indicated that at days 0-3 all of the challenged mice with 200 $LD_{50/ml}$ tetanus toxin in every group of samples (TTMA, TTMB , TTMC and TT) were dead. At day 7 three-tenth of mice in TT group were survived, while those immunized with TTMA, TTMB, and TTMC were all dead. Hence , TT had the shortest onset.

At day 15, five of TT immunized mice, two of TTMB immunized mice, and three of TTMC immunized mice were survived but none in TTMA immunized mice. From these results, they might show that the protective effect in TT immunized mice was increased,while those in TTMB and TTMC immunized mice just produced. Controversly, TTMA immunized mice still had no any propective power.

At Day 30, the protective effect continuously increased , the number of survived mice were more than the earlier time, as nine of TT immunized mice,three of TTMA immunized mice, seven of TTMB immunized mice and nine of TTMC immunized mice.

At day 45, the protective power of TT and TTMC were maximum, all immunized mice that be challenged with 200 $LD_{50/ml}$ tetanus toxin were survived. While five of TTMA and eight of TTMB immunized mice were survived.

Table 9 Number of survived mice which immunized with tetanus toxoid preparations.

Preparations / Days	TT	TTMA	TTMB	TTMC
0	0	0	0	0
3	0	0	0	0
7	3	0	0	0
15	5	0	2	3
30	9	3	7	9
45	10	5	8	10
60	8	10	10	10
75	7	10	10	10
90	3	10	9	7
105	0	10	10	3
120	0	10	10	1
140	0	8	10	0
160	0	7	10	0
180	0	5	10	0

* TT = Adsorbed tetanus toxoid.

TTMA = Tetanus toxoid microcapsule A.

TTMB = Tetanus toxoid microcapsule B.

TTMC = Tetanus toxoid microcapsule C.

At day 60 and 75 , all preparations of microcapsules, TTMA, TTMB and TTMC, could save the life of all ten of immunized mice but in TT immunized mice the quantity of survived mice decreased, only eight at day 60 and seven mice at day 75 were survived. And then decreased respectively, three mice at days 90 and none were survived at day 105 until 180.

It should be considered that the longest immunizing power of TT in protecting the immunized mice was only 90 days. The highest protective effect of TTMA was during day 60 until 120 and after that it decreased. But it still had partially protective effect until day 180 .

TTMB,could protect all of the immunized mice during days 60 until 180. The final preparaion in table 9 was TTMC. It had the most protective effect during day 45-75, all mice were saved ,after that they decreased. None mice of TTMC immunized were survived at day 140 and so on.

Therefore TT had the shortest onset(7 days) and also the shortest duration in immunizing activity (90 days). While TTMB had the longest duration (180 days) and the onset was longer than TT.(15 days)

Table 10 shows the results in potency testing of mixture of adsorbed tetanus toxoid (TT),the shortest onset and duration, and tetanus toxoid microcapsules, the longer onset and duration preparations, TTMA,TTMB and TTMC ratio 1:1 as TT+TTMA, TT+TTMB and TT+TTMC respectively.

For preparation of TT+TTMA, two-tenth of survived immunized mice were once found at day 7. The maximun effect in saving the mice life,all ten were not dead during days 30 and 120. After that no. of survived mice were decreased.

As these results, at the early time it might be the immunizing effect of TT. In the middle period,not only the immunizing activity of TT

Table 10 Number of survived mice which immunized with tetanus toxoid preparations.

Preparations / Days	TT+TTMA	TT+TTMB	TT+TTMC	PBS. pH 7.4
0	0	0	0	0
3	0	0	0	0
7	2	3	2	0
15	6	6	5	0
30	10	10	10	0
45	10	10	10	0
60	10	10	10	0
75	10	10	10	0
90	10	10	7	0
105	10	10	3	0
120	10	10	1	0
140	8	9	0	0
160	7	10	0	0
180	4	10	0	0

TT+TTMA = Adsorbed tetanus toxoid+Tetanus toxoid microcapsules A ratio 1:1.

TT+TTMB = Adsorbed tetanus toxoid+Tetanus toxoid microcapsules B ratio 1:1.

TT+TTMC = Adsorbed tetanus toxoid+Tetanus toxoid microcapsules C ratio 1:1.

PBS.pH 7.4 = Phosphate buffer saline solution pH 7.4.

but also the effect of TTMA that produced longer potency than single TT. In addition,the results of TTMB and TTMC in comparison of TT were in the pattern which similar to that of TTMA but the mixture of TT and TTMB was the longest. Hence, to prepared an effective tetanus toxoid preparation, the shortest onset and longest duration of action, mixing the TT and TTMB in the ratio 1:1 should be done.

Figure 18 shows the comparison between the number of survived mice in potency testing of adsorbed tetanus toxoid (TT), tetanus toxoid microcapsule A (TTMA) and mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsule A (TT+TTMA). There are significantly difference (table 11-13) in number of survived mice both among preparations ; TT, TTMA,and TT+TTMA; and period of times during day 0 to 30 (day 0 ,3 ,7 ,15 and 30)

But there are not significantly difference (table 14-15) in number of survived mice both among preparations and period of times during day 45 to 90 .(days 45, 60 ,75 ,and 90)

On the other hands (during day 105 to 180 number of survived mice are significantly difference only pairs of TT and TTMA , TT and TT+TTMA but TTMA and TT+TTMA are not significantly difference.(table 16-18)

Figure 19 shows the comparison between the number of survived mice in potency testing of adsorbed tetanus toxoid (TT) , tetanus toxoid microcapsules B (TTMB) and mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsule B (TT+TTMB).

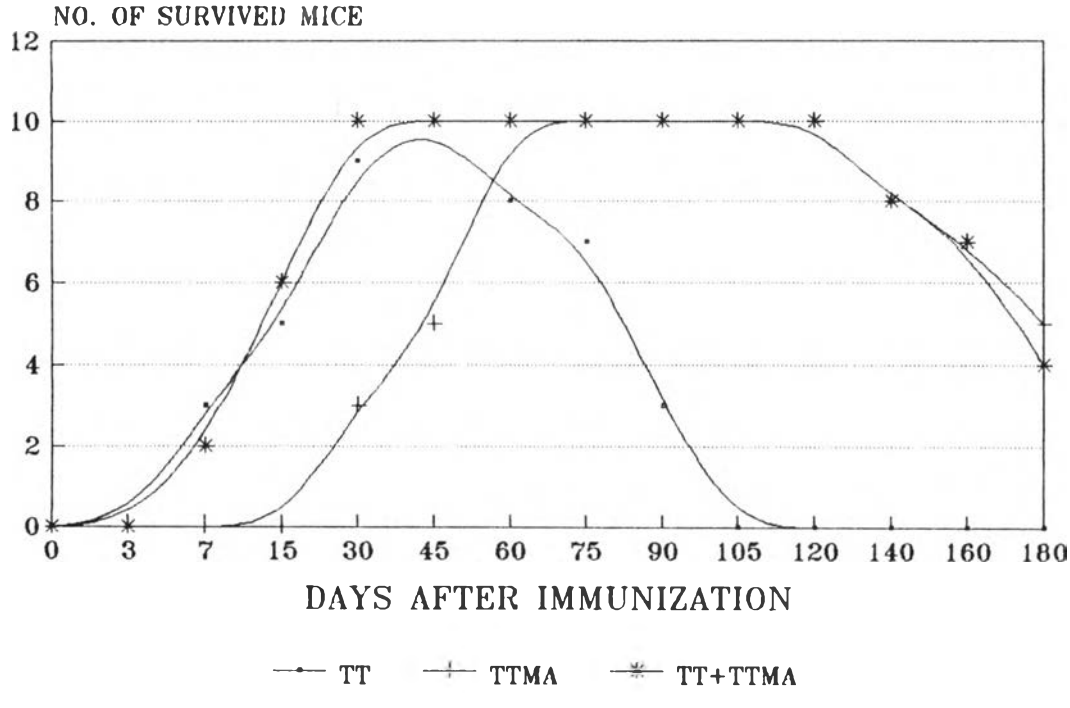

Figure 18 The comparison between number of survived mice in potency testing of TT , TTMA , and TT+TTMA.

Table 11 No. of survived mice in first month.

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT
0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
7	0	0	0	2	3	2	3
15	0	2	3	6	6	5	5
30	3	7	9	10	10	10	9

Table 12 ANOVA table of survived mice during days 0 and 30.

Source	df	SS	MS	F
Treatment	6	36.09	6.015	70.43
Block	4	343.55	85.8875	1005.71
Residual	24	2.05	0.0854	
Total	34	381.69		

Treatment; from table , $F_{0.05}(6,24) = 2.49$

$70.43 > F_{0.05}$

Block ; from table 24 , $F_{0.05}(4,24) = 2.76$

$1005.71 > F_{0.05}$

Table 13 Pairs of tetanus toxoid preparations that are significantly difference in no. of survived mice during days 0-30.

Preparation	TT	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC
TT	–	S	S	S	–	–	–
TTMA	–	–	–	–	–	–	–
TTMB	–	S	–	–	–	–	–
TTMC	–	S	S	–	–	–	–
TT+TTMA	–	S	S	S	–	–	–
TT+TTMB	–	S	S	S	–	–	–
TT+TTMC	–	S	S	S	–	–	–

Table 14 No. survived mice during days 45 and 90.

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT
45	5	8	10	10	10	10	10
60	8	10	10	10	10	10	10
75	10	10	10	10	10	10	7
90	10	9	7	10	10	3	3

Table 15 ANOVA table of survived mice during days 45 and 90.

Source	df	SS	MS	F
Treatment	6	22.00	3.6667	0.9059
Block	3	23.14	7.7133	1.9056
Residual	18	72.86	4.0478	
Total	27	118		

Treatment; from table 24, $F_{c.os}$ (6,27) = 3.87

0.9059 < $F_{o.os}$

Block ; from table 24, $F_{o.os}$ (3,27) = 3.10

1.9056 < $F_{o.os}$

Table 16 No. of survived mice during days 105 and 180.

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT
105	10	10	3	10	10	3	0
120	10	10	1	10	10	1	0
140	8	10	0	8	9	0	0
160	7	10	0	7	10	0	0
180	5	10	0	4	10	0	0

Table 17 ANOVA table of survived mice during days 105 and 180.

Source	df	SS	MS	F
Treatment	6	622.34	103.7233	80.0705
Block	4	26.11	6.5275	5.0389
Residual	24	31.09	1.2954	
Total	34	679.54		

Treatment; from table 24, $F_{0.05}$ (6,24) = 2.49

$80.0705 > F_{0.05}$

Block ; from table 24, $F_{0.05}$ (4,24) =2.75

$5.0398 > F_{0.05}$

Table 18 Pairs of tetanus toxoid preparations that are significantly difference in no. of survived mice during days 105-180.

Preparation	TT	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC
TT	–	–	–	–	–	–	–
TTMA	S	–	–	S	–	–	S
TTMB	S	S	–	S	S	–	S
TTMC	–	–	–	–	–	–	–
TT+TTMA	S	–	–	S	–	–	S
TT+TTMB	S	S	–	S	S	–	S
TT+TTMC	–	–	–	–	–	–	–

* S = Significantly difference

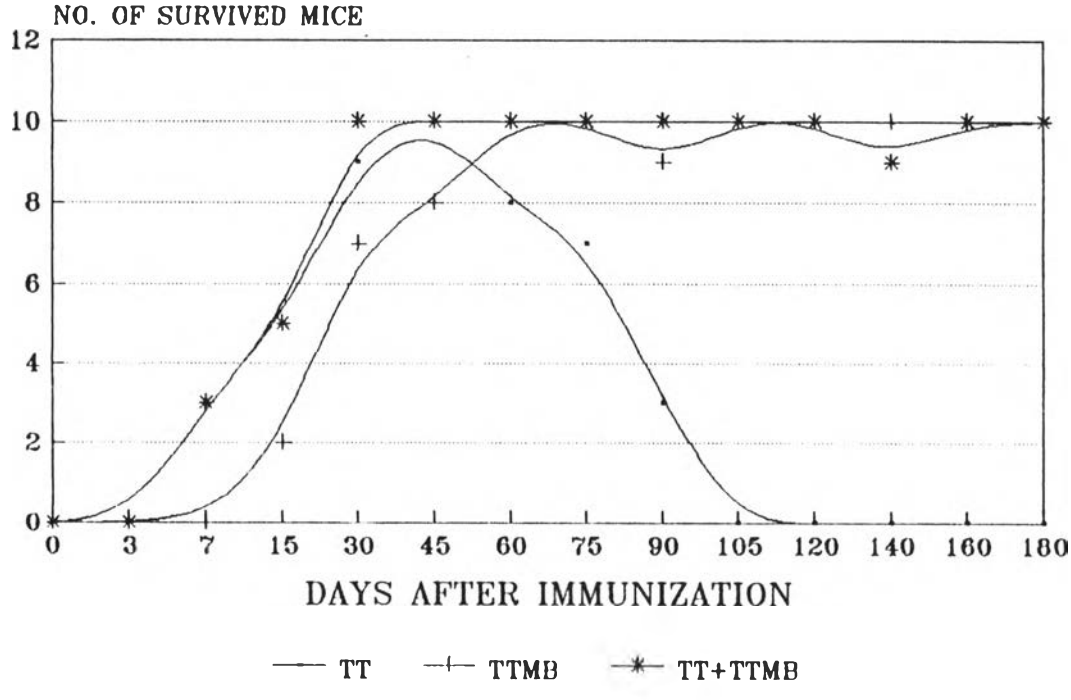

Figure 19 The comparison between number of survived mice in potency testing of TT , TTMB , and TT+TTMB.

During days 0 and 30 there are not significantly difference in no. of survived mice between TT and TT+TTMB but TT and TTMB, TTMB and TT+TTMB are significantly difference. (table 11-13)

During days 45 and 90 there are not significantly difference in no. of survived mice among these preparations.(table 14-15)

During days 105 and 180 no. of survived mice are not significantly difference in TTMB and TT+TTMB but TT and TTMB , TT and TT+TTMB are significantly difference.(table 16-18)

Thus order of immunization action of these three preparations TT , TTMB , and TT+TTMB ,are likely to that in figure 16 but TTMB and TT+TTMB had longer time of immunization action.

Figure 20 shows the comparison between the number of survived mice in potency testing of adsorbed tetanus toxoid (TT) ,tetanus toxoid microcapsule C (TTMC) and mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsule C (TT+TTMC). No. of survived mice during days and 30 are not significantly difference in TT and TT+TTMC but significantly difference in TT and TTMC ,TTMC and TT+TTMC. (table 11-13) During days 45-90 and 105-180 , there are not significantly difference in no. of survived mice among these preparations. (table 14-18)

According to these , although no. of survived mice during day 0-3 between TT and TTMC , TTMC and TT+TTMC are significantly difference but there are not significantly difference after days 45 until 180 . Thus , there are not advantage in using TTMC or TT+TTMC rather than TT.

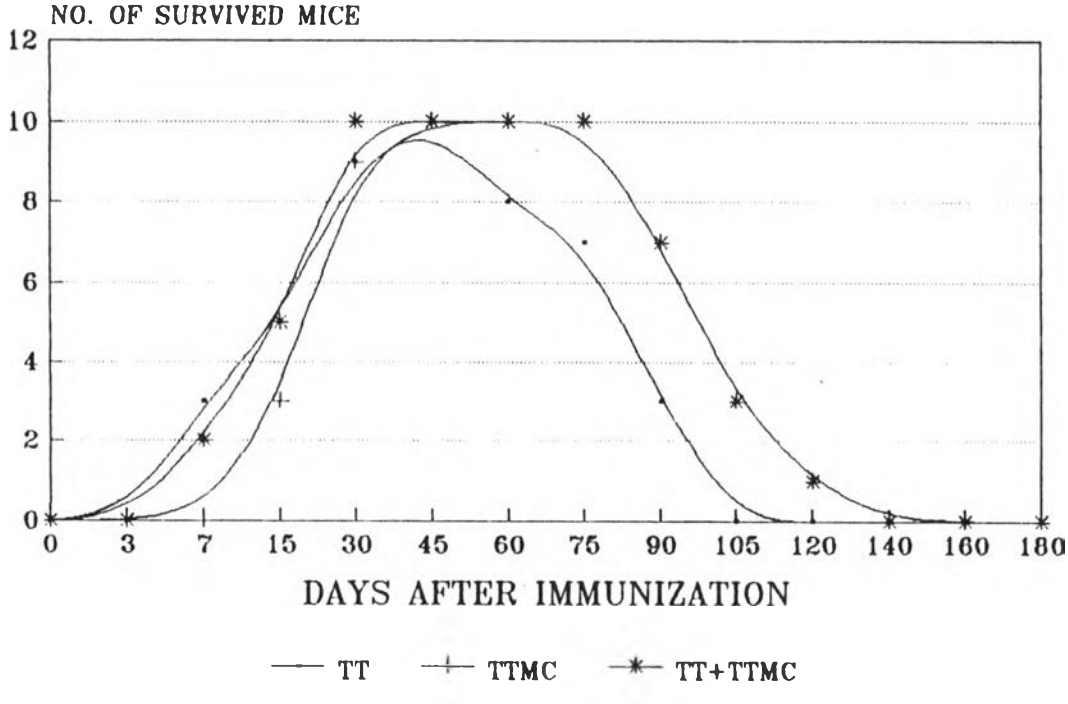

Figure 20 The comparison between number of survived mice in potency testing of TT , TTMC , and TT+TTMC.

Figure 21 shows the comparison between the number of survived mice in potency testing of adsorbed tetanus toxoid (TT) ,mixture of adborbed tetanus toxoid and tetanus toxoid microcapsule A (TT+TTMA) , mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsule B (TT+TTMB) , mixture of adsorbed tetanus toxoid and tetanus tixoid microcapsule C (TT+TTMC). During day 0-30 no. of survived mice are not significantly difference (table 11-13) between TT and TT+TTMA , TT and TT+TTMB , TT and TT+TTMC but they are significantly difference between all of the other pairs of these four preparations.

In contrast ,during days 45-90 ,there are not significantly difference (table 14-15) among these preparations. At the last period ,day 105-180, TT and TT+TTMC are also not significantly difference (table 16-18) in no. of survived mice.

As these preparations, TT+TTMB may be the best preparation that having the shortest onset likely to TT and the longest action likely to TTMB.

Figure 22 shows the conparison between the number of survived mice in potency testing of TTMA , TTMB and TTMC. All pairs all these preparations are significantly difference in no. of survived mice during days 0-30(table 11-13) and 105-180 (table 16-18) but there are not significantly difference in no. of survived mice during days 45 - 90. (table 14-15) The sequence in expressing the first protective effect are TTMC , TTMB and TTMA alternately. For the long acting preparation TTMB is the longest, next is TTMA and the last one is TTMC.

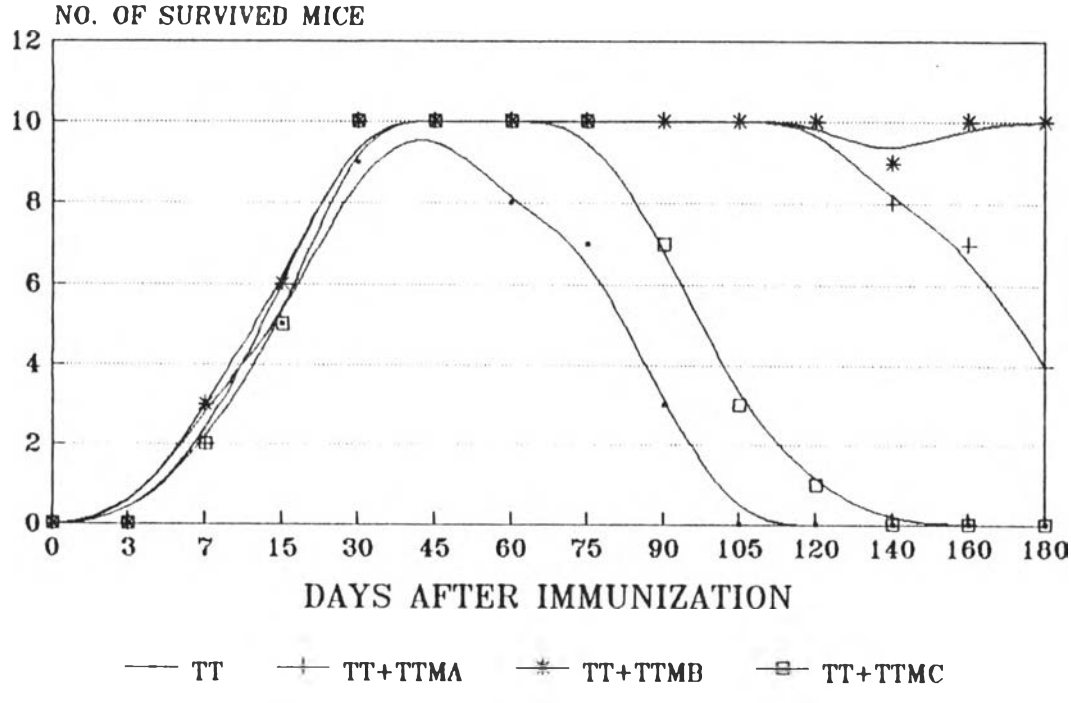

Figure 21 The comparison between number of survived mice in potency testing of TT , TT+TTMA , TT+TTMB , and TT+TTMC.

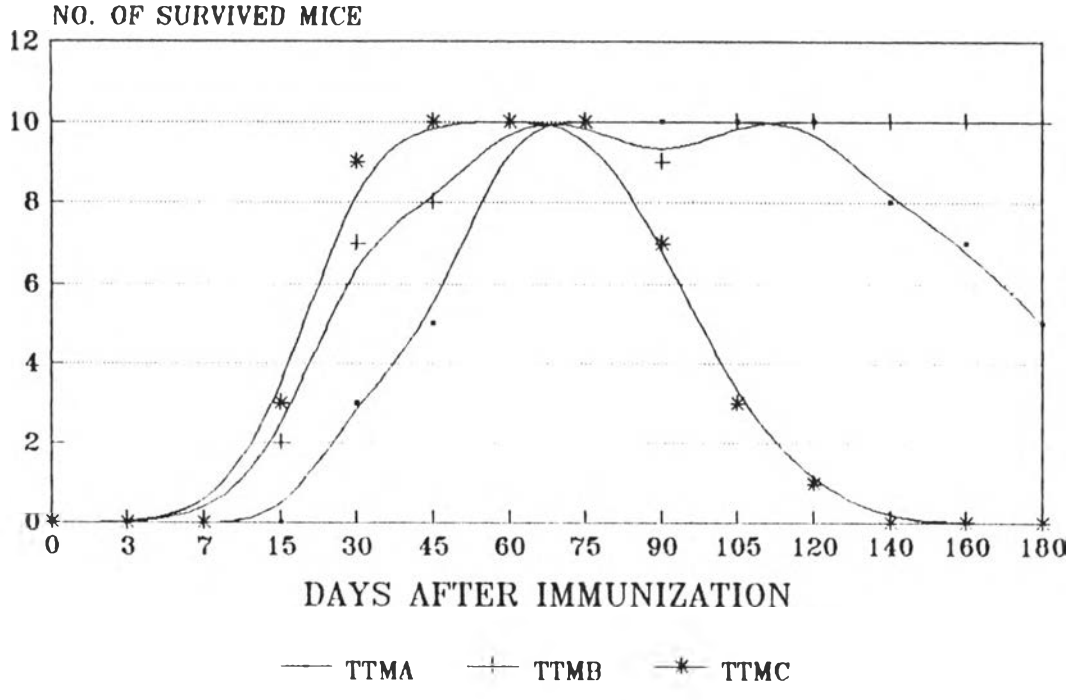

Figure 22 The comparison between number of survived mice in potency testing of TTMA , TTMB , and TTMC.

3.3) Antibody Determination

To determine the antibody levels or titers of tetanus toxoid preparationsin immunized mice, after each groups of mice were immunized, blood was withdrawed and serum was collected for quantitative analysis by passive hemagglutination technique. The antibody levels or titers were calculated relatively to standard tetanus immunoglobulin as unit/ ml.

Table 19 shows the mean titers that immunized with adsorbed tetanus toxoid (TT) ,Tetanus toxoid microcapsules A (TTMA), tetanus toxoid microcapsules B (TTMB) and tetanus toxoid microcapsules C (TTMC)in mice.

At day 0 , the mean titers of all preparation were at zero point. At day 7,that of TT was 0.13 u/ml ,the most and the first titers to be found. The second was TTMC ,0.07 u/ml .while that of TTMA and TTMB were still 0.0 u/ml. After that titers of every preparations increased . TT's titers were highest at day 45-75 and then rapidly decreased to zero at day 140.

TTMA, the titers were at zero levels until day 7 , then stepwise increased to maximum level (2.00 u/ml) at day 90 and decreased to 0.78 u/ml at day 180. TTMB, had the maximum titers (3.40 u/ml) at day 75 - 90 and then slightly decreased to 1.2 u/ml at day 180. TTMC, the titers rapidly increased from 0.75 u/ml (days 15) to 2.00 u/ml in 15 days then they were increased to the highest level (3.4 u/ml)at day 60 and after that decreased until 0.4 u/ml at day 180.

Table 20 shows titers that immunized with tetanus toxoid preparations, (TT+TTMA, TT+TTMB and TT+TTMC) in mice. Titers of every

Table 19 The mean titers in mice that immunized with TT,TTMA,TTMB and TTMC.

Preparations	Titers (unit/ml)			
Days	TT	TTMA	TTMB	TTMC
0	0.00	0.00	0.00	0.00
3	0.13	0.00	0.00	0.07
7	0.63	0.00	0.25	0.45
15	1.00	0.06	0.65	0.75
30	1.40	0.10	1.80	2.00
45	2.10	0.45	2.60	2.80
60	2.00	0.75	3.20	3.40
75	2.00	1.80	3.40	1.00
90	0.55	2.00	3.40	1.00
105	0.45	1.70	2.40	0.60
120	0.06	1.20	2.10	0.25
140	0.00	0.95	1.85	0.45
160	0.00	0.85	1.50	0.40
180	0.00	0.78	1.20	0.40

TT = Adsorbed tetanus toxoid.

TTMA = Tetanus toxoid microcapsule A.

TTMB = Tetanus toxoid microcapsule B.

TTMC = Tetanus toxoid microcapsule C.

Table 20 The mean titers in mice that immunized with TT+TTMA,TT+TTMB,TT+TTMC,and PBS pH 7.4 .

Preparations	Titers (unit/ml)			
Days	TT+TTMA	TT+TTMB	TT+TTMC	PBSpH7.4
0	0.00	0.00	0.00	0.00
3	0.15	0.12	0.15	0.00
7	0.80	0.85	0.80	0.00
15	1.60	1.60	1.60	0.00
30	2.60	2.60	2.80	0.00
45	3.20	3.40	3.40	0.00
60	3.20	3.40	2.50	0.00
75	3.20	2.80	1.50	0.00
90	2.20	2.50	0.85	0.00
105	1.70	2.20	0.50	0.00
120	1.10	1.90	0.40	0.00
140	0.95	1.40	0.40	0.00
160	0.85	1.10	0.20	0.00
180	0.80	1.20	0.20	0.00

TT+TTMA = Adsorbed tetanus toxoid+Tetanus toxoid microcapsules A ratio 1:1.

TT+TTMB = Adsorbed tetanus toxoid+Tetanus toxoid microcapsules B ratio 1:1.

TT+TTMC = Adsorbed tetanus toxoid+Tetanus toxoid microcapsules C ratio 1:1.

PBS.pH 7.4 = Phosphate buffer solution pH 7.4.

preparations start to showe at day 3. The levels were similar to that of TT. Next to this time , all of the titers level all increased to the highest level at day 45 (3.2-3.4 u/ml). They are at a steady-state for a while , then differently decreased , TT+TTMC was the rapidest decreased preparation (from 3.4 u/ml at day 45 to 0.85 u/ml at day 90 ,0.4 u/ml at day 120 and then 0.2 u/ml at day 160-180.)

The second one was TT+TTMA , titers at the highest level was 3.2 u/ml during day 45-77 , 1.7 u/ml at day 105 , 0.95 u/ml at day 140 , and then to the lowest level, 0.80 u/ml at day 180. The last one was TT+TTMB ,at 3.4 u/ml titers at the higest level wasduring day 45-60 and slowly decreased to 1.1-1.2 u/ml at day 180.

Figure 23 shows the comparison between titers (u/ml) that immunized with adsorbed tetanus toxoid (TT) , tetanus toxoid microcapsules A (TTMA) and mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsules A (TT+TTMA) in mice and detected by passive hemagglutination technique.

Day 0-30 , titers level (u/ml) of TT were similar to that of TT+TTMA but that of TTMA were significantly lower.(table 21-23) Day 45-90, titers level (u/ml) of all preparations were significantly difference. The highest level of TT were similar to that of TTMA but they were not at the same time, TT was at day 45 but TTMA was at days 90. (table 24-26) Day 105-180, the levels of TTMA and TT+TTMA were alike but that of TT were significantly less than the other two. (Table 27-29)

Hence, the titers of tetanus toxoid preparations immunized in mice that expressed in early period (day 0-30) of TT+TTMA should be the

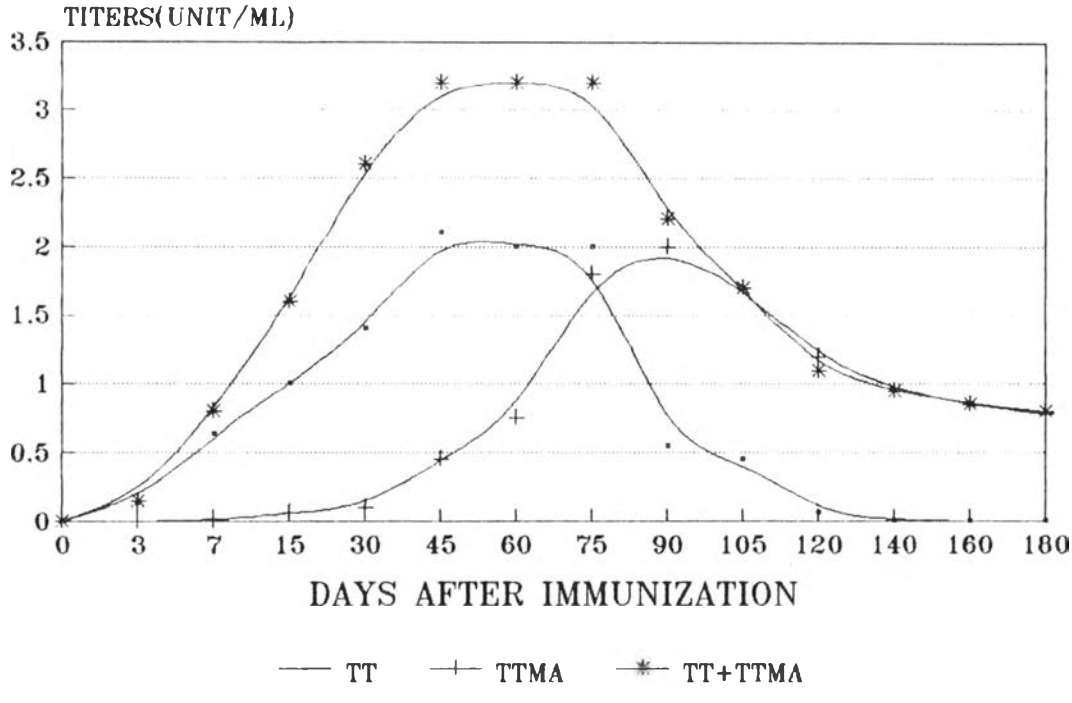

Figure 23 The comparison between titers in mice that immunized with TT , TTMA , and TT+TTMA.

Table 21 Mice's titers in first month.

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.07	0.15	0.12	0.15	0.13
7	0.00	0.25	0.45	0.80	0.85	0.80	0.63
15	0.06	0.65	0.75	1.60	1.60	1.60	1.00
30	0.10	1.80	2.00	2.60	2.60	2.80	1.40

Table 22 ANOVA table of Mice's titer during day 0-30

Source	df	SS	MS	F
Treatment	6	4.2136	0.7023	4.3486
Block	4	17.0957	4.2739	26.4638
Residual	24	3.8749	0.1615	
Total	34	25.1842		

Treament; from table 24 , $F_{0.05}(6,24) = 2.49$

$4.3486 > F_{0.05}$; Reject the null hypothesis ($H_{1\ 0}$)

Block; from table 24 , $F_{0.05}(4,24) = 2.76$

$26.46381 > F_{0.05}$; Reject the null hypothesis ($H_{1\ a}$)

Table 23 Pairs of tetanus toxoid preparations that are significantly difference in titers of mice during days 0-30.

Preparation	TT	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC
TT	–	S	–	–	–	–	–
TTMA	–	–	–	–	–	–	–
TTMB	–	–	–	–	–	–	–
TTMC	–	S	–	–	–	–	–
TT+TTMA	–	S	–	–	–	–	–
TT+TTMB	–	S	–	–	–	–	–
TT+TTMC	–	S	–	–	–	–	–

* S = Significantly difference

Table 24 Mice' s titers during days 45 and 90.

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT
45	0.45	2.60	2.80	3.20	3.40	3.40	2.10
60	0.75	3.20	3.40	3.20	3.40	2.50	2.00
75	1.80	3.40	2.00	3.20	2.80	1.50	2.00
90	2.00	3.40	1.00	2.20	2.50	0.85	0.55

Table 25 ANOVA table of Mice's titer during day 45-90

Source	df	SS	MS	F
Treatment	6	17.27	2.8783	15.7975
Block	3	4.55	1.5167	8.3244
Residual	18	3.28	0.1822	
Total	27	25.1		

Treament; from table 43 , $F_{0.05}(6,18) = 2.60$

$15.7975 > F_{0.05}$;Reject the null hypothesis ($H_{1\ 0}$)

Block; from table 43 , $F_{0.05}(3,18) = 3.10$

$8.32441 < F_{0.05}$; Accept the null hypothesis ($H_{1\ .}$)

Table 26 Pairs of tetanus toxoid preparations that are significantly difference in titers of mice during day 45-90.

Preparation	TT	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC
TT	–	–	–	–	–	–	–
TTMA	–	–	–	–	–	–	–
TTMB	S	S	–	S	–	–	S
TTMC	–	S	–	–	–	–	–
TT+TTMA	S	S	–	S	–	–	–
TT+TTMB	S	S	–	S	–	–	S
TT+TTMC	–	S	–	–	–	–	–

* S = Significantly difference

Table 27 Mice's titers during days 105 and 180.

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT
105	1.70	2.40	1.80	1.50	2.20	1.50	0.45
120	1.20	2.10	1.10	0.85	1.90	0.85	0.06
140	0.95	1.85	0.80	0.50	1.40	0.05	0.00
160	0.85	1.50	0.45	0.40	1.10	0.40	0.00
180	0.78	1.20	0.40	0.43	1.50	0.43	0.00

Table 28 ANOVA table of Mice's titer during day 105-180

Source	df	SS	MS	F
Treatment	5	8.9261	1.4877	17.0608
Block	4	3.6558	0.9139	10.4811
Residual	24	2.0938	0.0872	
Total	34	14.6757		

Treament; from table 43 , $F_{0.05}(6,24) = 2.49$

$17.0607 > F_{0.05}$; Reject the null hypothesis ($H_{1\ 0}$)

Block; from table 43 , $F_{0.05}(4,24) = 2.75$

$10.4811 > F_{0.05}$; Reject the null hypothesis ($H_{1\ .}$)

Table 29 Pairs of tetanus toxoid preparations that are significantly difference in titers of mice during days 105-180.

Preparation	TT	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC
TT	–	–	–	–	–	–	–
TTMA	S	–	–	–	–	–	–
TTMB	S	S	–	S	S	–	S
TTMC	S	–	–	–	–	–	–
TT+TTMA	S	–	–	–	–	–	–
TT+TTMB	S	–	–	S	S	–	S
TT+TTMC	S	–	–	–	–	–	–

* S = Significantly difference

immunizing activity of TT, in middle period, the titers were so higher that they should be the immunizing activity of TT and the adjuvanity of TTMA . In final period (day 105-180) they should be the immunizing activity of long acting tetanus toxoid microcapsules A (TTMA).

Figure 24 shows the comparison between titers (u/ml) that immunized with TT, TTMB ,and TT+TTMB in mice,and detected by passive hemagglutination technique. Day 0-30 , titers level of these three preparations were not significantly difference. (table 21-23) Day 45-90 and 105-180 , the titers level of TT+TTMB were nearly to that of TTMB but that of TT were significantly lower than the other two. (table 24-29)

It should be concluded that TTMB had a proper diameter range to be used to show a good immunizing activity that made a short onset similar to that of TT but could prolong the action until 180 day or longer.

Figure 25 shows the comparison between titers that immunized with TT ,TTMC ,and TT+TTMC in mice and detected by passive hemagglutination technique. Day 0-30 , the titers level of all three preparations were not significantly difference , while that of TTMC and TT+TTMC had tendency to increase to the higher level than that of TT.

Day 45-90 and 105-180, titer levelof TTMC were significantly likely to that of TT+TTMC , on the other hand , that of TT were significantly lower than the other two. The reasons were similar to that of figure 24.

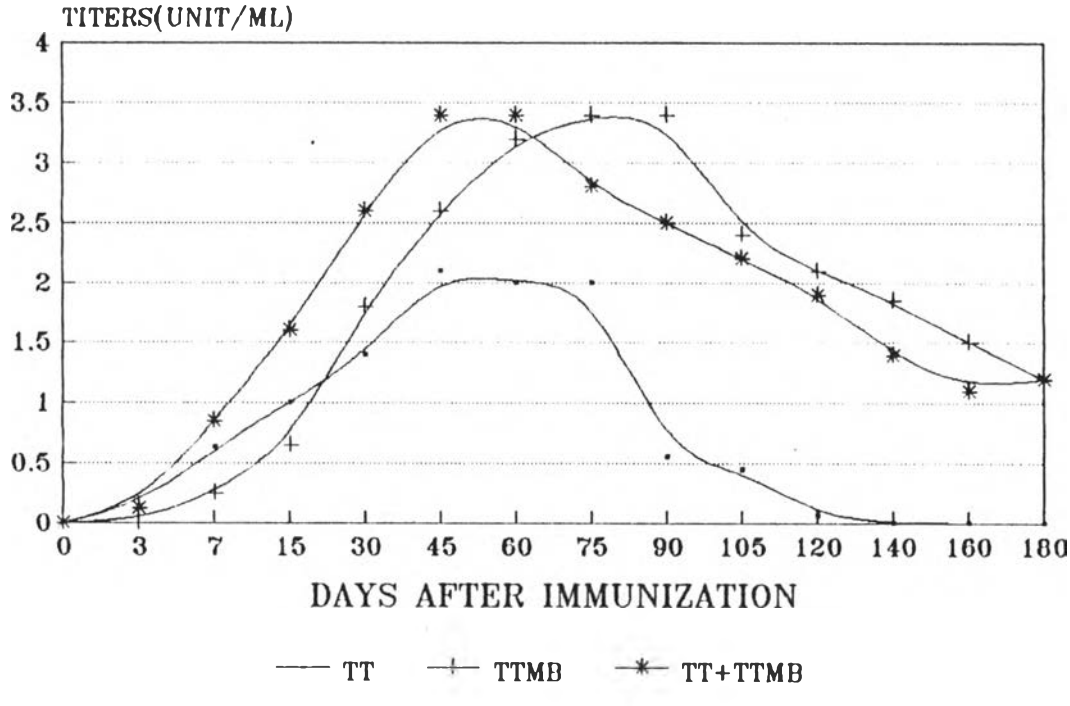

Figure 24 The comparison between titers in mice that immunized with TT , TTMB , and TT+TTMB.

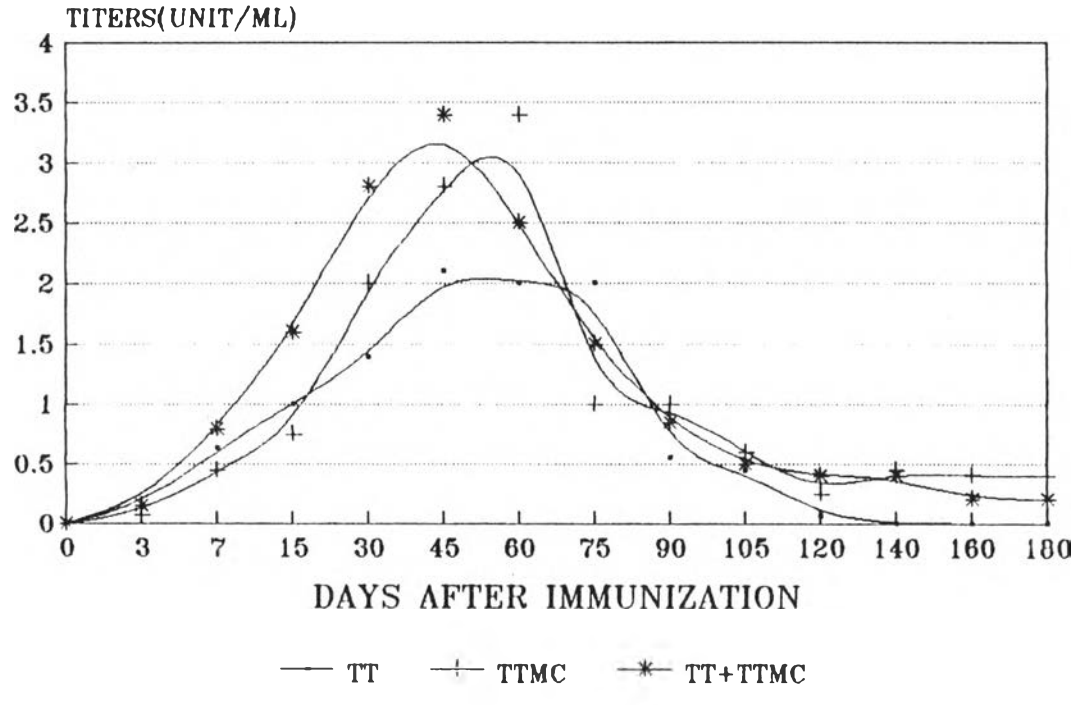

Figure 25 The comparison between titers in mice that immunized with TT , TTMC , and TT+TTMC.

Figure 26 shows the comparison between titers that immunized with TT+TTMA ,TT+TTMB , and TT+TTMC in mice and detected by passive hemagglutination technique. Day 0-45 the titers of these preparations were the same but they differently decreased, TT+TTMC was the rapidest , the second was TT+TTMA and the slowest was TT+TTMB.

3.4) Correlation in Potency testing and antibody levels

According to the results (table 9-10 and 19-20) ,the titer levels at the same time as the potency testing that could survived all ten mice. When all of challenged mice were survived, the tites levels were 2.1-2.0 unit/ml in TT immunized mice, 1.2-1.8 unit/ml in TTMA immunized mice, 2.6-1.2 unit/ml in TTMB immunized mice, 2.0-1.0 unit.ml in TTMC immunized mice, 3.2-1.1 in TT+TTMA immunized mice, 3.4-1.2 in TTMB immunized mice and 3.4-1.50in TT+TTMC immunized.

In addition, in order to survive all mice from tetanus toxin, the titers increased higher than 2.0 unit/ml. After the highest titers level, the titers decreased while all of the mice still safe. Most of the mice died when the titers levels were lower than 1.1 unit/ml. At earlier time the protective titers were higher than that of in the final time. The reason was discussed. In consideration , Immunoglobulin M (IgM) is the main immunoglobulin produced early in the primary response. When the cells contact with the tetanus toxoid. The serum antibody concentration was high (2.1 unit/ml) and continued to rise for 30 days. After these, it declines and drop to very low level (0.55 unit/ml).

In the facts, the first antibody appeared is IgM followed by immunoglobulin G (IgG) . IgM levels tend to decline earlier than IgG

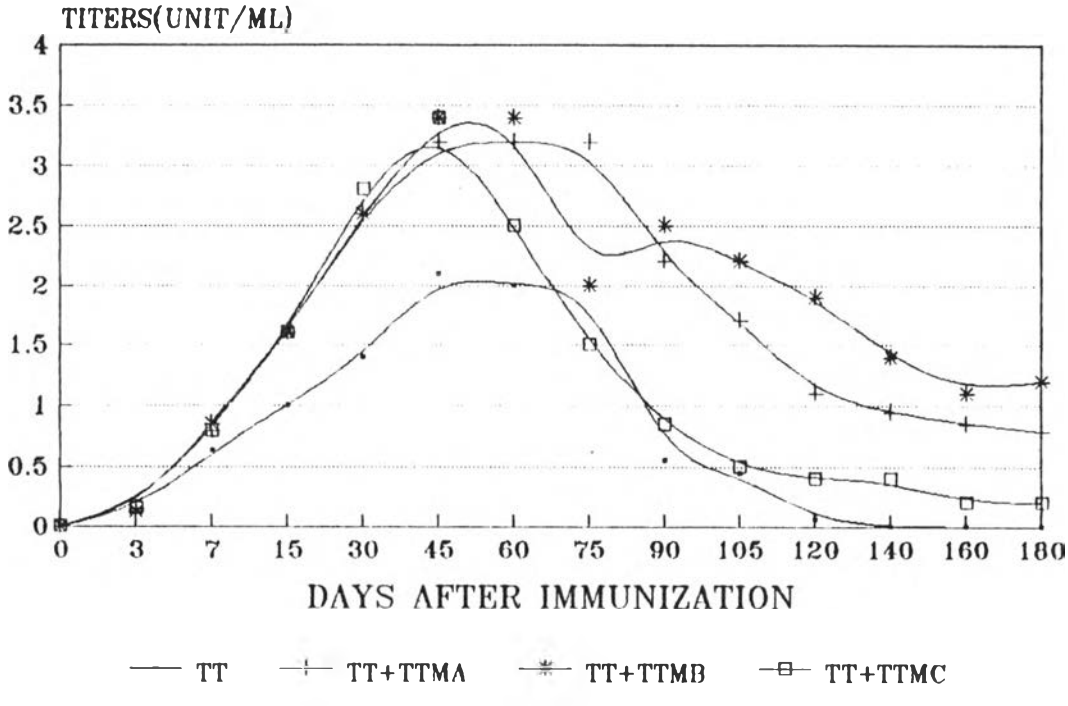

Figure 26 The comparison between titers in mice that immunized with TT , TT+TTMA , TT+TTMB , and TT+TTMC.

levels. So,at the earlier period the immune response was due to the concentration of IgG and IgM. Then IgM decline and only IgG was the mian remained. And according to Stanly ,et al.(1988),IgG is more specific in neutralizing tetanus toxin than IgM. Although titers levels were lower than 2.0 units/ml, it still had protective effect.When the titers decreased lower than 1.0 units/ml , they could not safe the mice furthermore.

The Prolong Action of Tetanus Toxoid Microcapsules

Because of tetanus toxoid were encapsulated in microcapsules, thus it could not immediately induce immune response. So it take time to diffuse through the polymeric membrane. That caused tetanus toxoid microcapsules had a longer immunizing on set. After that tetanus toxoid was slowly released and kept the level for a long period. Resulted in prolonging the immunizing activity of tetanus toxoid.

Moreover,microcapsules that injected subcutaneously or intramuscularly behaved slowly clearance from the injection site are also produced long action of tetanus toxoid.

The Immune Response in the Mixture of Tetanus Toxoid and Tetanus Toxoid Microcapsules

In case of combination of tetanus toxoid and tetanus toxoid microcapsules , there are many advantages in immunizing effects. First, the onset was early as same as tetanus toxoid. The second, a longer duration of action likely to that of tetanus toxoid microcapsules. And the third, the maximun titers was higher than tetanus toxoid or tetanus

toxoid microcapsules alone.

The reason was that tetanus toxoid acted as the first antigen that resulted in early immunizing effect. Tetanus toxoid microcapsules was the second antigen. So there is a rapid antibody response to higher level than the primary immune response from tetanus toxoid alone. This is attribute to the antigen sensitive "memory cells" after the first contact with tetanus toxoid. During this period, the amount of IgM produced is similar to that after the first contact with tetanus toxoid alone. However a much larger amount of IgG antibody is produced and the level tend to persist much longer than in the primary response.

Influencing of Size Distribution on Titers

Difference of size distribution of tetanus toxoid microcapsules produced difference in expressing of antibodies. The largest mean vesicle size of TTMA should produce the longest duration of immunization but the results were not same as expected. TTMA had the shorter immunizing activity than TTMB which had the smaller mean vesicle sizes.

TTMA was obtained from 2000 rpm centrifugation. At this low speed traces of lecithin could also easily be pelleted as same as large microcapsules. Therefore this portion may compose of the mixture of lecithin traces and larger microcapsules that could hardly expect the results whether they could prolong the immunizing activity.

TTMB,the second portion that was obtained by 5000 rpm centrifugation occupied more uniformity size than TTMA. There was not any trace of lecithin because it was already precipitated in the first portion. So TTMB is unique tetanus toxoid microcapsules which could

release tetanus toxoid uniformly. Resulted in the high immune response.

The last portion of TTMC that obtained from 12000 rpm centrifugation. It take much time to collect only small amount of tetanus toxoid microcapsules. These TTMC could protect the mice in a short period,during day 30-75 only. The reason was also discussed. Most particle size of this portion are small that effected the stability of microcapsules. According to the amount of surface area are converted-related to tha size of vesicle. The smaller vesicle size was , the more surface area occupied. The polymeric membrane of microcapsules could be destroyed by the attacking of phospholipase. So the small microcapsules could easily be attacked and destroyed by phospholipase. Resulted in a short action of immunization than other portions of tetanus toxoid microcapsules.

In addition, because of TTMC had much surface area,most encapsulated tetanus toxoid microcapsules could easily pass through the membrane and acted as the primary antigen likely to TT alone but then the immune response could not persist for a long time.

Further Experiments

For further experiments, reproducing for much more yield in 5000 rpm centrifugal size range may obtained. Lecithin may separate out at lower speed of centrifugation and the small amount of microcapsules from the third portion may discarded.