Chapter II

Materials and Methods

Materials

1) Adsorbed tetanus toxoid. [G.P.O.]

2) Benzidine. [Zigma]

3) Carboxymethyl chitin. [Asahi Kasie Chemical Industries,LTD,Tokyo]

4) Purified egg yolk lecithin.[Asahi Kasie Chemical Industries,LTD,Tokyo]

5) Plained tetanus toxoid [G.P.O.]

6) Sheep red blood cell.[N.I.H.]

7) Standard tetanus immunoglobulin. [N.I.H.]

8) Tetanus toxin.[G.P.O.]

9) Citric acid.[E.Merk's , A.R.Grade.]

10) Dextrose anhydrous.[E.Merk's, A.R.Grade.]

11) Dibasic sodium phosphate. ($Na_2HPO_4.12H_2O$) [E.Merk's, A.R.Grade.]

12) Dichloromethane.[E.Merk's, A.R.Grade.]

13) Formalin.[E.Merk's .A.R.Grade.]

14) Hydrochloric acid.[E.Merk's .A.R.Grade.]

15) Magnesium sulfate.[E.Merk's, A.R.Grade.]

16) Monobasic Potassium phosphate. ($Na_2HPO_4.12H_2O$) [E.Merk's , A.R.Grade.]

17) Monobasic sodium phosphate. ($NaH_2PO_4.H_2O$)
[E.Merk's ,A.R.Grade.]

18) Sodium azide.[E.Merk's ,A.R.Grade.]

19) Sodium chloride.[E.Merk's ,A.R.Grade.]

20) Sodium citrate.[E.Merk's , A.R.Grade.]

21) Sodium nitrite.[E.Merk's , A.R.Grade.]

* G.P.O. = The Goverment Pharmaceutical Organization .

**N.I.H. = The National Institutes of Health.

Instruments

1) Analytical balance.[Sartorious, 1602 MP 8-1]

2) Scanning electron microscope .[Joel ,JSM-T220A]

3) Glass wares. (Pyrex)

4) Homoginizer [Ultra-turrax T 25]

5) Magnetic Stirrer.[Skalenwert ,T 20/1]

6) Micropipette.[Gilson, H 25000 L]

7) Microscope.[Olympus]

8) Multichannel micropipette.[Socorex]

9) Microtiter plates.(U-bottom)

10) pH meter. (Orion research , 611]

11) Particulate count instrument,Coulter counter.
[Hyad/Royco ,300]

12) Refigerated centrifugation.[Backman,J 2-21]

13) Vaccum deposition caoter [Balzers,SCD 040]

14) Vortex mixer.[Lab-line instruments, 1291]

15) Water bath.[Julabo]

Methods

1) Preparation of lecithin and carboxymethyl chitin walled tetanus toxoid microcapsules. (Kato,Arakawa,and Kondo,1984).

Purified egg yolk lecithin was dissolved in dichloromethane at 50 mg/ml . Carboxymethyl chitin (CMC) was dissolved in a phosphate buffer solution (pH 7.4) at a concentration of 0.2 percent (w/v). To 10 ml of tetanus toxoid was added an equal volume of the lecithin solution, and the mixture was vigorously agitated by a vortex mixer for 30 second to give a w/o emulsion. The emulsion obtained was quickly added with stirring to 100 ml of CMC solution to yield a w/o/w emulsion. After 10 minutes stirring, another 100 ml of the CMC solution was added to the complex emulsion under stirring and the stirring was further continued for 24 hours until the dichloromethane was completely evaporated out.In this preparation, small vesicles of tetanus toxoid microcapsules were suspended in phosphate buffer saline solution pH 7.4. In this experiment, the larger scale , 400 ml.of tetanus toxoid was obtained.

2) Separation of Various Vesicle Size of tetanus toxoid microcapsules by centrifugation Method. (Tyle,1990)

Lecithin and carboxymethyl chitin walled tetanus toxoid microcapsules was centrifuged at 2,000 rpm for 15 minutes,the precipitation were washed three time with phosphate buffer solution pH 7.4 (PBS pH 7.4) the precipitate was weighted and redispersed in PBS pH 7.4 . Thus , this preparation was collected for preparation of TTMA. The supernatant was continued to treatment by centrifugation at 5,000 rpm 20 minutes and 12000 rpm 30 minutes,respectively. The precipitation were washed and collected in the same way as the TTMB ,and TTMC,the final supernatant was discarded. The concentration of the preparation was adjusted by 16.5 % weight per volume in phosphate buffer solution pH 7.4 (PBS pH 7.4). Thus three preparation of tetanus toxoid microcapsules were obtained;

2.1) TTMA = Tetanus Toxoid Microcapsules A.

2.2) TTMB = Tetanus Toxoid Microcapsules B.

2.3) TTMC = Tetanus Toxoid Microcapsules C.

Then mixed tetanus toxoid and tetanus toxoid microcapsules as below;

2.4) TT+TTMA = Mixture of Tetanus Toxoid and Tetanus Toxoid Microcapsules A ratio 1:1 v/v.

2.5) TT+TTMB = Mixture of Tetanus Toxoid and Tetanus Toxoid Microcapsules B ratio 1:1 v/v.

2.6) TT+TTMC = Mixture of Tetanus Toxoid and Tetanus Toxoid Microcapsules C ratio 1:1 v/v.

3) Testing the Quality of the Tetanus Toxoid Microcapsules Preparations.

3.1 Physical Testing.

a) Particle Size Analysis of the Microcapsules

A coulter counter (Hyad/Royco model 300) was used to determine the microcapsules diameter in samples from formulations TTMA, TTMB ,and TTMC. A microscopic method counting 1 ml. was adopted for these diluted 1:1000 samples. this allowed the diameters to be measured.

b) Scanning Electron Microscopy

This technique was used to study the surface and shape characteristics of microcapsules. The dry sample of microcapsules was coated with gold, using a vacuum deposition coater (Balzers,SCD 040) The thickness of the coating was dependent on the geometry of the sample and was obtained on a trial and error basis.

The sample were photographed by magnifying photograph times in the electron microscope (Joel, JSM-T 220A) and photographing.

3.2) Animal Testing

a) $LD_{50/ml}$

Tetanus toxin is 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 PBS pH 7.4 ,0.5 ml of each dilutions shall be inject subcutaneously into 10 mice 17-20 g of weight/mice. and then observed for 5 days. Calculated for accumulated died value-D,accumulated survived value-S, accumulated mortality ratio and $LD_{50/ml}$.

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

$-\text{Log } LD_{50/ml}$ =Log dilution above 50 % mortality + proportionate distance

b) Immunization

Mice, *Swiss albino* , inbreeding ,2400 males 17-20 gm of weight were divided to 8 groups by sampling method, 0.5 ml volume of each of the preparations was injected subcutaneously to 10 of each groups of mice.

8 preparations for injection were

1) Tetanus toxoid microcapsules collected from2,000 rpm (TTMA) centrifu ation.

2) Tetanus toxoid microcapsules collected from 5,000 rpm. (TTMB) centrifugation.

3) Tetanus toxoid microcapsules collected from 12,000 rpm. (TTMC) centrifugation.

4) Adsorbed tetanus toxoid.(TT)

5) PBS.pH 7.4

6) Mixture of tetanus toxoid and tetanus toxoid microcapsules A ratio 1:1 (TT+TTMA).

7) Mixture of tetanus toxoid and tetanus toxoid microcapsules B ratio 1:1 (TT+TTMB).

8) Mixture of tetanus toxoid and tetanus toxoid microcapsules C ratio 1:1 (TT+TTMC).

c) Potency Testing (Mittal ,Jaiswal and Gupta,1979)

10 mice of each group of the imminuzed mice were challenged with 200 LD_{50} of tetanus toxin at day 0,3,7,14,30,45,60,75,90,105,120,140, 160,and 180. The quantity of survived mice on the fifth day were recorded. The results of each groups were compared by an adequate statistical method.

d) Antibody Determination (William,1997;Stites,1982)

Blood was withdrawn from the mice 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 heat inactivated serum in saline with 1% normal rabbit serum(heat inactivated and absorbed with sheep erythrocytes)were made in microtiter plates. A 50 microlitres amount of suspension of indicator erythrocytes was mixed with 50 microlitres of serum dilution. The PHA was read after 16 hours and the recipocol value of the greatest dilution still giving agglutination was taken as the titer.

Passive Hemagglutination test (PHA)

It is possible to determine of antibody by PHA mothod. PHA reaction is composed of sensitized red blood cell with tetanus toxoid and tetanus antibody. We can see as hemagglutination in the test. An antibody titer of test serum is calculated as a relative titer against the reference antibody.

Handling process of test

1) Preparation of 10 % Fixed Sheep Red Blood Cell(FSRC)by formalin.

2) Preparation and check up of Absorbed Normal Rabbit Serum (NRS).

3) Preparation of Diluent.

4) Preparation of Antigen Sensitized Sheep Red Blood Cell (SSRC) .

a) Diazotization of Benzidine.

b) Washing of formalin fixed sheep red blood cell.

c) Sensitization of tetanus toxoid to FSRC

d) Washing of sensitized sheep red blood cell and suspend to make final 2 %.

5) Procedure of Antibody Determination by PHA method.

a) Dilution of reference serum and test serum.

b) Addition of sensitized sheep red blood cell. (SSRC)

c) Keeping at room temperature for a night.

6) Judgement.

Explanation of Each Process of PHA Test

1 Preparation Method of 10% Fixed Sheep Red Blood Cell (FSRC) by Formalin.

a) Take a sheep blood same volume to Alserver in injector.

b) The blood is filtrated by some sheets of gauze and then 3 times volume of saline is added into the filtrated blood.

c) The diluted blood is washed 5 times by centrifugation at 3000 rpm for 5 minutes.

d) After final centrifugation for washing the supernatant is removed gentle, and then the sediment of red blood cell is measured its

volume.

e) The diluent which is adjusted to pH 7.2 with 0.01 N NaOH solution is added to the sediment of red blood cell to make final 8% of suspension.

f) Same volume of 3% formaldehyde in saline(pH 7.2) is added like a drop by drop into 8% of sheep red blood cell suspension with mild agitation and then the sheep red blood cell are fixed with mild agitation at 37°C for 3 hours. (3%formaldehyde solution is prepared from formalin including 37% formaldehyde by 12 times dilution with saline)

g) Furthermore, the mixed sheep red blood cell suspension is incubated at 37°c for a night without agitation.

h) The next day, the fixed sheep red blood cell (FSRC) are washed 5 times with saline by centrifugation.

i) After final centrifugation for washing,the supernatant is remove and then sediment of red bloodcell is measured its volume.

j) Finally, the FSRC is adjusted to make 10% suspension in saline which include 0.05% sodium azide.

2 **Preparation and Check up of Absorbed Normal Rabbit Serums (NRS).**

a) Take a blood from rabbit which is normal on against tetanus. Blood is pooled in a Roux bottle and then incubated at 37° c for 2 to 3 hours on the horizontal position and then the Roux bottle is kept at cold room for a night.

b) On the next day, the serum is taken out by decantation. After that the serum is isolated from blood by centrifugation at 2,000 rpm for 10 minutes.

c) A small amount of this serum is used for testing to know a suitable way to make a absorbed normal rabbit serum as follow,two different ways.

1) Using this serum after inactivation 56°C 30 minutes for absorption with fixed sheep red blood cell.

2) Using this serum without inactivation for absorption with fixed sheep red bloods cell.

Absorption method of normal rabbit serum.

a) 0.1 ml of 10% fixed sheep red blood cell (FSRC) is taken in a bottle.

b) 5 ml of saline is added into the bottle and then the FSRC is washed by centrifugation at 2,000 rpm for 5 minutes.

c) After discarded of the supernatant, 1 ml of NRS is added into the sediment (FSRC) with agitation.

d) The mixture is incubated at 37°C for one hour with agitation at several times.

e) After centrifugation at 2,000 rpm for 5 minutes,the supernatant is taken out to another bottle and kept as Absorbed Normal Rabbit Serum (ANRS).

d) We have to decide the best way from the results of preliminary test for heagglutination.

e) The remained NRS is treated by the sameway which was getting thebest result on the preliminary test. After this, the ANRS is used for routine work.

3) Preparetion of Diluent.

This diluent is used for dilution of anti-serum and also washing of sensitized red blood cell.

Composition of diluent.

Absorbed Normal Rabbit Serum(ANRS)	1.00 ml.
$MgSO_4.7H_2O$	0.20 gm.
NaCl	0.85 gm.
Sodium azide (NaN_3)	0.05 gm.
Distilled water to make	100.00 ml.

4) Preparation of Antigen Sentitized Sheep Red Blood Cells (SSRC).

a) Diazotization of Benzidine.

1) 0.23 gm of Benzidine is dissolved in 50 ml. of 0.25 N. HCl with agitation in ice-bath .....solution (1).

2) 0.175 gm of $NaNo_2$ is dissolved in 5 ml of cold distilled water .....solution(2).

3) The both solution are kepted at 0 C to 2 C in ice-bath.

Notice: Following process must be performed under condition to keep at 0C to 2 C in ice-bath.

4) 5 ml of the solution(2) is added more slowly (1-2 ml /min) into 50 ml of the solution(1) with agitation.

5) After addition of all solution(2) (5 ml),the mixture is agitated for one minute every 5 minutes interval for 30 minutes.

6) This reacted mixture must be used immediately within 30 minutes.

b) Washing of formalin fixed sheep red blood cell.

1) 5 ml. of 10% FSRC is suspended in 40 ml. of saline in centrifugation.

2) After centrifugation at 2,000 rpm for 5 minutes, the supernatant is discarded and the sediment is wash again with saline.

3) 1.0 ml of 0.11 M. PBS pH 7.2 is added into the packed cell to make 50% of FSRC suspension.

c) Sensitization of tetanus toxoid to FSRC.

1) 30 ml. of cold toxoid which is prepared on adjusted into 30 Lf/ml concentration with 0.11 M PB.pH7.2,is added to 1 ml of 50% FSRC.

2) 1 ml.of BDB solution indicated at 3.1 is added into 14 ml.of 0.11 M.PB. pH7.2 which is kept at 0 C to 2 C,and then the mixture is poured into antigen prepared above 3.1 very quickly.

3) The mixture is kept at room temperature for 10 minutes.

d) Washing of sensitized sheep red blood cell and resuspensing to make final 2%.

1) After centrifugation at 2,000 rpm for 5 minutes the sediment is washed two times with 40 ml.of diluent by centrifugation.

2) Packed cell is resuspended to 25 ml of diluent to make 2% of antigen sensitizedsheep red blood cell,and then kept at 4 C.

5) Procedure of Antibody Determination by PHA Method.

a) Test serum must be absorbed with fixed sheep red blood cell before use.

1) Test serum is diluted in 5 times with 0.05% sodium azide added saline (0.1ml:0.4ml).

2) Diluted test serum is inactivated at 56 C for 30 minutes.

3) 0.025 ml of 20% Fixed Sheep Red Blood Cell (FSRC) which was washed two times with saline by centrifugation is added to inactivated test serum for absorption.

4) The mixture is incubated at 37° C for one hour with agitation some times.

5) After centrifugation at 2,000 rpm for 5 minutes,the supernatant is taken out as a test serum.

b) Original dilution of the test sample and reference serum.

c) Two fold dilution of diluted reference serum and test serum. 0.025 ml of reference serum and test serum are put into wells of A row of microtitration plate of U-bottom type respectively. After that they are diluted to two fold dilution with diluent.

d) Some quantity of sensitized sheep red blood cell which been keeping at cold room,is washed with diluent by centrifugation and the sediment is resuspended in diluent to make final 0.2% cell suspension. After that 0.075 ml of cell suspension is added to each well of diluted reference serum and test serum on the microtitration plate by dropper respectively.

e) The plate is shaked for 3 minutes.

f) The plate is kept for a night at room temperature and then antibody titer is determined as a relative titer by the hemagglutination pattern of reference serum and test serum.

6) Calculation of Antibody Titers

$$TU = RU \times TD$$

RU = Unit of reference antibody which was determined by hemagglutination pattern.

TD = Multiple of dilution of test sample which was determined by hemagglutination pattern.

TU = Unit of test sample

4) STATISTICAL ANALYSIS

4.1) The results from pyrogen testing and antibody detemination were filled in contingency table as below;

Table 3 Contingency table

Days	TTMA	TTMB	TTMC	TT+TTMA	TT+TTMB	TT+TTMC	TT	$\Sigma x_{.j}$	$\Sigma x_{.j}^2$	$x_{.j}$
$\Sigma x_{i.}$										
$\Sigma x_{i.}^2$										
$x_{i.}$										

4.2) The hypothesized the results of potency testing and antibody determination as below;

Hypothesis

Pyrogen Test

Treatment.

$H_{A\ o}$ = There are not significantly difference in no. of survived mice among tetanus toxoid preparation.

$H_{A\ a}$ = There are significantly difference in no. of survived mice among tetanus toxoid preparation.

Block.(time)

$H_{B\ o}$ = There are not significantly difference in no. of survived mice in each period of time.

$H_{B\ a}$ = There are significantly difference in no. of survived mice in each period of time.

Antibody Determination

Treatment.

$H_{A\ o}$ = There are not significantly difference in mice,s titers among tetanus toxoid preparations .

H_{A} a = There are significantly difference in mice's titers among tetanus toxoid preparations

Block.(time)

$H_{B\ o}$ = There are not significantly difference in mice's titers in each period of time.

H_0 = There are significantly difference in mice titers in each period of time.

4.3) The data from contingency table were calculated.

CT. (Correction Term) = $\frac{(\Sigma x_{..})^2}{N}$

SS_{total} (Total sum of squares) = $\Sigma x_{ij}^2 - CT$

SS_{block} (Block sum of squares) = $\frac{\Sigma(\Sigma x_{.j}^2)}{n_j} - CT$

$SS_{treatment}$ (Treatment sum of squares) = $\frac{\Sigma(\Sigma x_{i.}^2)}{n_i} - CT$

MS (Mean square) = SS/df

$F_{(Treatment)}$ = $\frac{\text{Treatment mean square}}{\text{Error mean square}}$

$F_{(Block)}$ = $\frac{\text{Block mean square}}{\text{Error mean square}}$

Degree of freedom of total = $(n_i . n_j) - 1$

Degree of freedom of block = $n_i - 1$

Degree of freedom of treatment = $n_j - 1$

degree of freedom of error = $(n_i - 1).(n_j - 1)$

4.4) Established the ANOVA table.

Table 4 ANOVA table

Source	df	SS	MS	F
Treatment Block Residual				
Total				

4.5) Compared the F value from table 43 and the calculated F value from ANOVA teble . Then justment to accept or reject the null hypothesis.(H_o)

4.6) If results of the test conclude that there were significantly difference between the treatments or the block,"Duncan's New Multiple Range Test" was used to determine which pair of the test were difference.