CHAPTER II

Materials and Methods

Materials

1) Adsorbed tetanus toxoid., G.P.O.
2) Tetanus toxin., G.P.O.
3) Chitin., Batch No. CHAWO30601, Seikagaku kogyo Co., Ltd.
4) Purified egg yolk lecithin., Asahi Kasie Chemical Industries, LTD, Tokyo.
5) Methanol AR., Batch No. 19462346, E. Merck.
6) Hydrochloric acid AR., Batch No. 18574432, E. Merck.
7) Absolute ethanol AR., Batch No. K19462283, E. Merck.
8) Isopropyl alcohol AR., Batch No. K17894034, E. Merck.
9) Acetone AR., Batch No. K18185270, BDH.
10) Dichlorometane AR., Batch No. K17379450, E. Merck.
11) Chloroform AR., Bacth No. K13659145, E. Merck.
12) Sulphuric acid AR., Batch No. K15376432, E. Merck.
13) Polyoxyethylene sorbitan monolaurate AR., Bach No. 12 HO159, Sigma.
14) Sodium hydroxide pellets AR., Batch No. C788298, E. Marck.
15) Dibasic sodium phosphate AR., Batch No. A578486, E. Merck.
16) Monobasic sodium phosphate AR., Bacth No. 432468, E. Merck.
17) Sodium chloride AR., Batch No. K13445704, E. Merck.
18) Sodium carbonate AR., Batch No. A759192, E. Marck.
19) Sodium hydrogen carbonate AR., Batch No. K16289329, E. Merck.

20) Citric acid anhydrous AR., Batch No. 27488, Fluka.

21) Monobasic potassium phosphate AR., Batch No. 471687, E. Merck.

22) Potassium chloride AR., Batch No. TA 915536, Carlo Erba.

23) Sodium monochloroacetate AR., Batch No. 312265/1293, Fluka.

24) Peroxidase-goat anti-mouse IgG (H+L) Batch No. 21011754, Zymed.

25) Albumin bovine farction V powder (BSA) Batch No. 10H0257 , Zymed.

26) Orthophenylene diamine (OPD) Batch No. 31216790, Zymed.

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

All Chemicals were analytical or pharmaceutical grades and were used as received.

Equipments

1) Analytical Balance, Sartorius GMPH, Range 300 g/mg, Germany.
2) pH Meter, Model SA 520, Orion, USA.
3) Scanning electron microscope, Joel, JSM-35 CF.
4) Homogenizer, Ultra-turrax T 25.
5) Microscope, Olympus Optical Co., Ltd.
6) Micropipette, Gilson, H 25000L.
7) Magnetic Stirrer, Skalenwert, T 20/1.
8) Refrigerated centrifugation, Beckman, J 2-21.
9) Vacuum deposition coater, Balzers, SCD 040.
10) Vortex mixer, Scientific Industries. Inc.
11) Thermostatted Shaker Bath, Julabo v SW 1, Juchheim Labortechnik HG, West Germany.
12) Multichannel micropipette, Finnpipette.
13) Biorad, model 450 microplate reader.
14) Glass ware, Pyrex.
15) Microtiter plate, Nune, Denmark.

Methods

1) Preparation of carboxymethylchitin. (Kato, Arakawa, and Kondo, 1984).

Flaky chitin (Nanyo Kasei chemicals, Ltd., Tokyo) was washed with a methanolic HCL solution to remove the coloured materials, and the decolorized chitin was washed three times with ethanol and dried. The dried chitin had been mixed thoroughly with NaOH solution, the mixture was stored in a refrigerator to make it swell. The swollen chitin was added with stirring to sodium monochloroacetate in isopropanol solution, and the temperature was kept at 4°C for 2 hours ; the reaction was then allowed to proceed at room temperature for 24 hours. At the end of this period, isopropanol was removed by filtration and the reaction product, carboxymethylchitin, was dissolved in 1500 ml of deionized water. The solution was neutralized with HCL solution and dialysed against deionized water for 48 hours. Acetone was added to the dialysed solution to separate carboxymethylchitin and the precipitated polymer was dried and pulverized. The chemical structure of carboxymethylchitin is shown in figure 6.

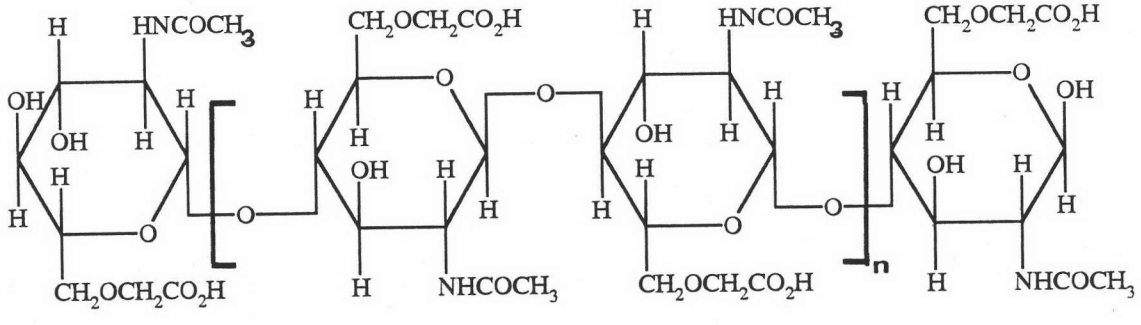

Figure 6 Chemical structure of carboxymethylchitin

2) Preparation of lecithin and carboxymethylchitin walled tetanus toxoid microcapsules. (Kato, Arakawa, and Kondo, 1984).

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 a vortex mixer for 30 second 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 out. Lecithin and carboxymethylchitin walled tetanus toxoid microcapsules were centrifuged at 15,000 rpm for 30 minutes, the precipitation was washed three times with phosphate buffer solution pH 7.4. The precipitate was weighed and redispersed in phosphate buffer solution. The concentration of the preparation was adjusted by 16.5% weight per volume in phosphate buffer solution pH 7.4. In this experiment, the large scale, 1,200 ml of tetanus toxoid was prepared.

In this experiment, preparation of drugs were stored in a refrigerator at $4^{\circ}C$ for 0, 3, 6 and 9 months.

Preparation of drugs for injection as following :

2.1) Tetanus Toxoid Microcapsules = TTM

2.2) Mixture of Tetanus Toxoid and Tetanus Toxoid Microcapsules ratio 1 : 1 v/v. = TT + TTM

2.3) Tetanus Toxoid = TT

2.4) Phosphate Buffer Solution pH 7.4 = PBS pH 7.4

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

3.1) Physical Testing.

a) Particle Size Analysis of the Microcapsules

The microcapsules diameter in samples from formulation TTM, was measured from the photographed of Scanning Electron Microscopy. Number of particle size must have at least 625 particles to be measured the diameter.

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. (Balzer, 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 - 35 CF) and photographing.

3.2) Animal Testing

a) $LD_{50/ml}$

Tetanus toxin was diluted to concentration of 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. A volume of 0.5 ml of each dilution was injected subcutaneously into each of 10 mice (17-20 g /

mouse) and mice were observed for 5 days. Accumulated died value-D, accumulated survived value-S, accumulated mortality ratio and $LD_{50/ml}$ were calculated.

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

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

$LD_{50/ml}$ was the concentration of the toxin that produced 50% mortality in mice injected with 0.5 ml toxin subcutaneously.

b) Immunization

Mice, Swiss albino, inbreeding, 3840 males (17-20 mg /mouse weight/mice) were divided to 240 mice per group by sampling method, 0.5 ml volume of each of the preparations that were stored for 0, 3, 6 and 9 months was injected subcutaneously into mice.

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

Ten mice of each group of the immunized mice were challenged with 200 $LD_{50/ml}$ of tetanus toxin at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180. The number of survival were recorded in five days. The results of each group was compared by using statistical method.

d) Antibody Titer Determination

Ten mice of each group of the immunized mice were bled by heart puncture technique at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180 after each immunization. Serum was separated by centrifugation, aliquoted and stored at -20°C. The result of each group was compared by using statistical method.

The presence of antibodies was determined by ELISA.

Reagents used for ELISA

1. Buffers

a) Carbonate / Bicarbonate Buffer Solution (Coating Buffer) 50 mM pH 9.6 Na_2Co_3 (1.59 gm), $NaHCO_3$ (2.93 gm), and distilled water (1 L).

b) Phosphate Buffer Saline with Tween 20-PBST (Washing Buffer). PBS pH 7.4, NaCl (8.0 gm), KH_2PO_4 (0.2g), Na_2HPO_4 .$12H_2O$ (2.9 gm), KCl (0.2 gm), Tween 20 (0.5 ml), and distilled water (1 L).

c) Citrate Buffer (Substrate Buffer, pH 5.0). Citric acid (10.3 gm), Na_2HPO_4 .$2H_2O$ (18.16 gm), and distilled water (1 L).

2. Preparation of Blocking Solution. 3% bovine serum albumin (BSA) in PBST.

3. Preparation of Diluent Buffer. 1% BSA in PBST.

4. Preparation of Conjugate. Horseradish peroxidase conjugated to goat anti-mouse immunoglobulin G 1 : 10 in diluent buffer.

5. Substrate Solution. O - Phenylene diamine (60 mg), and substrate buffer.

6. Stopping Solution. 4 N H_2SO_4

ELISA procedure

Wells of polystyrene microtiter plates were coated with tetanus toxoid dilution 1 : 10 in carbonate - bicarbonate coating buffer (100 µl/well). Plates were covered and left overnight at 4°C to allow the coating antigen adsorb to the wells. The plates were washed four times with PBS - tween buffer (PBST) and blotted to remove excess buffer. Blocking solution was added to each well (100 µl/well). The

plates were covered, incubated for 1 hr at 37°C and then washed four times with PBST and blotted. Serial five-fold dilutions (1:10, 1:50, 1:250, 1:1,250, 1:6,250, 1:31,250) of serum samples were prepared in diluent. One hundred microliters of each dilution was added into triplicate wells. Diluent buffer was used for control by adding 100 µl/well. After the incubation at room temperature for 1 hr, the plates were washed four times with PBST and blotted. One hundred microliters of a 1:4,000 dilution of goat anti-mouse IgG-HRP conjugate was added to each well and the plate was covered and incubated for 1 hr at room temperature and the plates were washed four times after incubation. Substrate solution O-phenylene diamine solution was prepared and 100 µl was added to each well. After 10-15 min of incubation at room temperature in the dark the reaction was stopped by adding 50 µl of 4 N sulfuric acid to each well. The plates were gently shaked before absorbance were measured at 492 nm with Biorad model 450, microplate reader (Davis and Gregodiadis, 1987). The diagram of procedure was shown in Figure 5.

4) Statistical Analysis

4.1 The results from this experiment were analyzed by two way analysis of variance (ANOVA).

The results of potency testing and antibody titer determination was tested as follow :

Hypothesis

- Potency test

Treatment (tetanus toxoid preparation)

H_{1O} : There are no significant difference in number of survived mice among tetanus toxoid preparations.

H_{1A} : There are significant difference in number of survived mice among tetanus toxoid preparations.

Block (period of time)

H_{2O} : There are no significant difference in number of survived mice at each time period.

H_{2A} : There are significant difference in number of survived mice at each time period.

- Antibody titer determination

Treatment

H_{1O} : There are no significant difference in titer level among tetanus toxoid preparations.

H_{1A} : There are significant difference in titer level among tetanus toxoid preparations.

Block

H_{2O} : There are no significant difference in titer level at each time period.

H_{2A} : There are significant difference in titer level at each time period.

Stability Testing

- Potency test

Treatment

H_{1O} : There are no significant difference in number of survived mice at each month storage period of same preparation.

H_{1A} : There are significant difference in number of survived mice at each month storage period of same preparation.

Block

H_{2O} : There are no significant difference in number of survived mice at each time period.

H_{2A} : There are significant difference in number of survived mice at each time period.

- Antibody titer determination

Treatment

H_{1O} : There are no significant difference in titer level at each month storage period of same preparation.

H_{1A} : There are significant difference in titer level at each month storage period of same preparation.

Block

H_{2O} : There are no significant difference in titer level at each time period.

H_{2A} : There are significant difference in titer level at each time period.