CHAPTER III

MATERIALS AND METHODS

Materials

1. Human Volunteers

Twenty healthy volunteers (11 men and 9 women) ranging in age 20-50 years, who had not been previously vaccinated with oral typhoid vaccine, were the subjects of this study.

We divided these volunteers into two groups, 11 persons, were vaccinated with Berna (Vivotif®), an oral commercial typhoid vaccine which is produceฦ by Swiss Serum and Vaccine Institue , Switzerland. The other group (9 persons) were vaccinated with oral typhoid vaccine, that was made by Dr. B.L. Reynolds, Thai Red Cross Society.

From each group of volunteers , collected five specimens of heparinized blood, serum, saliva, stool and intestinal lavage, before and after vaccination at 1, 2, 3, and 4 weeks and then every month for 3 months.

2. Vaccines

The oral typhoid vaccine consisted of the live

S.typhi auxotroph Ty 21a.

2.1 Vivotif ® Berna, is produced commercially by the Swiss Serum and Vaccine Institute, Berne. One pack comprises three enteric-coated white capsules, which are resistant to gastric juice, but release their contents in the more alkaline condition in the small intestine. Each capsule has stated to contain at least 10^9 live Ty 21a organisms.

2.2 Thai Red Cross Vaccine; the oral live attenuated typhoid vaccine (Ty 21a) was kindly supplied by Prof.Dr.R.Germanier, Swiss Serum and Vaccine Institute, Berne, Swizerland. The standard seed of S.typhi Ty 21a strain was kindly given by Dr.B.L.Reynolds, Thai Red Cross Institute, Thailand. As the gelatin capsule, was not resistant to gastric juice, it was necessary to administer sodium bicarbonate solution orally prior to each dose. Before use a colony count showed that at least $2x10^9$ live Ty 21a were present per capsule.

3. Microtitre Plates

The polystyrene microtitre plates " Nunc Immunoplate I , 4 - 39454 " were used as the solid phase support in the ELISA.

4. Polyethylene petridishes

The disposible polyethylene petridishes " Nunc with 5 cm in diameter " were used in the leukocyte migration inhibition test in agarose technique.

Methods

1. Vaccination Schedule

Shortly before the first vaccine dose (day 0) the heparinized blood, serum, saliva, stool and intestinal lavage were collected from the two group of volunteer and stored at -70° c.

The vaccine was administered orally according to the standard protocol on day 1, 3, 5. Since, the Vivotif ® Berna vaccine was in enteric-coated capsules, sodium bicarbonate (used to neutralized gastric acid) was not nescessary. But the Thai Red Cross Vaccine was presented in plain gelatin capsules, which should be sensitive to gastric acid, the 2.0 g of sodium bicarbonate were given orally, 2 minutes before vaccination to protect the live organisms in capsule from being destroyed by gastric acidity.

2. Collection and Storage of Specimens

2.1 Heparinized blood for LMI

Ten milliliters of blood were collected into a polyethylene syringe, then transfered into a screw capped tube containing 250 IU heparin and mixed well.

2.2 Serum

Blood samples were collected by vein puncture, the blood was allowed to clot at 37° c for 30 mins and the sera were separated. The sera were divided into small alliquots and stored at -70° c until used.

2.3 Intestinal Lavage

The procedure used for collection of the specimens was the one originally used for cleansing the intestine prior to contrast radiography (100). This was modified later for use in a study of intestinal immunity by Sack and co-worker (101).

Briefly, the subjects were asked to drink a large volume (approximately 2 -3 liters) of an isotonic balance salt solution (see appendix I), which contained no nutrients. Lavage was consumed at a rate of approximately one liter per 40 minutes. Drinking was stopped when the rectal effluent was judged relatively clear and free of particulate matter. Specimens of approximately 500 ml watery stool each were collected over a period of two hours. Each specimen was inactivated immediately in water bath at 56° c for 15 minutes, then filtered through a gauze and chilled in

an ice bath. The filter was centrifuged at 3,000 rpm for 1 hour, then the supernatant was collected for concentration (approximately 10-50 folds), by polyethyleneglycol (PEG). The concentrated specimens were divided into small aliquots and stored at -70° c until used.

2.4 Saliva

Saliva samples were obtained according to the method of Ostergaard and Blom (102).

Samples were collected 2 hours post prandially, when there is least variation in salivary flow rate and proteins. Following collection, the saliva was frozen at -20° c for 24 hours, thawed and then cellular debris and mucus was removed by centrifuge at 3,000 rpm for 30 minutes. The clear supernatant of saliva was aspirated and divided into small alliquots and stored at - 70° c until used.

2.5 Stool Extract

Stool extract samples were obtained according to the method of Evans (103) and Locarnini (104) with some modification.

The night before collection of the specimens, 30 ml of castor oil was administered.The morning, stool specimens were collected and held at 4° c for 4-6 hours, the wet weight of each stool specimen was

determined and sterile glycerol was added to final concentration of 20% followed by rapid freezing and storage at -20° c to minimize protease digestion of faecal immunoglobulin. Stool extract was prepared by suspending each thawed stool specimen in cool stool extract buffer (see appendix I) the final ratio was 1:5 (w/v). Stool suspension were agitated vigorously in an ultraturax mixer for 30 seconds and then centrifuged at 3,000 rpm for 1 hour at 4° c , the clear supernatant was then aspirated. Finally the stool extract was divided into small alliquots and stored at -70° c until used.

3. Preparation of antigen

Crude lipopolysaccharide (CLPS) that we used as the antigen was kindly supplied by Dr.B.L.Reynolds. It was the CWE from Salmonella typhi strain Ty2, the method that used to extract was phenol-water method originally by O'Neil and Todd (105).

10 gram acetone dried cells stirred for 3 hours with 100 ml of o.25 N trichloracetic acid at 2-4 °c. Centrifuged, wash with 100 ml of water and recentrifuged. Extracted with 45 % phenol at 56 °c for 15 mins. Seperated aqueous phase was dialysed with distilled water over night and then centrifuged at about 4000 g to remove debris ; freeze dry the supernatant.

4. Leukocyte Migration Inhibition Test (LMI)

The method of leukocyte migration inhibition test (LMI) followed that of Clasusen (106), Koonakosit (107) and Astor (108).

After collected the heparinized blood. High molecular weight dextran 6 % in saline was added in a ratio of 4 parts of blood to 1 part dextran and allowed to sediment at 37°c for 1 hr. The buffy coat was removed, centrifuged at 220xG for 5 mins, and the cell pellets washed three times in Hank's balance salt solution. The buffy coat was resuspended in medium 199 containing 10 % horse serum, 100 μnit/ml of penicillin, 100 μg/ml of streptomycin, 3 mM HEPES which adjusted the pH to 7.2-7.4 by 10 % sodium bicarbonate and the cell adjusted to a concentration of $2.2x10^{6}$ cells/ml.

Agarose medium was freshly prepared each day. A 1.2 % solution of agarose was prepared with sterile distilled water and the solution was autoclaved. When the solution had cooled to 50° c it was mixed 1:1 with medium 199 (10x) and supplemented to achieve a final concentration of 10 % horse serum, 100 unit/ml of penicillin, 100 μg/ml of streptomycin and 3 mM HEPES. This resulted in a pH of 7.2 to 7.4 after incubation of agarose plates. Six milliliters of the agarose were poured into disposable plastic petri

dishes and 12 holes were cut in the medium with stainless punch 2.0 mm in diameter.

The cells were preincubated with antigen (CLPS)) as follows : 20 ul aliquots of the cell suspension were placed in glass tubes and incubated at 37°c for 30 mins without antigen (controls) or with one of antigen. The optimum concentration of CLPS antigen in the test was 100 µg/ml. After incubation, 7 µl of each cell suspension were placed in the wells that had been cut in the agarose. Each specimen was run in triplicate and if more than one plate was used for testing an individual, control cells, incubated without antigen, were incubated on each plate.

The petri dishes were then incubated for 18 hrs at 37°c in an atmosphere of 5 % carbon dioxide and 95 % air saturated with water vapor, before measurement of migration and calculation of migration index (M.I.).

Migration index (M.I.) can be calculated from the formula : M.I. = Mx/Mo ; Mx = average areas of migration in test suspension ; Mo = average areas of migration in control suspension.

5. ELISA

5.1 Method of Coupling Antibody to Enzyme

The technique as described by Engvall and

Perlman (99) and was developed with some modification by Avrameas (110,111). This method is called one step glutaraldehyde technique. The enzymes that used were alkaline phosphatase (sigma type VII-S) and urease (sigma type VII, purified powder).

Alkaline phosphatase and urease containing 10.5 mg protein was dialysed against 0.1 phosphate buffer pH 6.8 overnight, and after that mixed with purified rabbit anti-human α- chain containing 5.0 mg protein and mixed well. The total volume of the mixture was adjusted to 2.5 ml with phosphate buffer, pH 6.8 and dialysed against phosphate buffer pH 6.8 with two changes of buffer. After that, 50 µl of 1 % glutaraldehyde (EM grade) prepared in 0.1 M of PBS 7.2 was added to dialysed protein mixture while it was stirred gently. The coupling reaction was allowed to proceed for 3 hours at room temperature. After coupling 0.1 ml of lysine solution pH 7.0 was added, after 2 hours the mixture was dialysed overnight in the cold against PBS 7.0, with three changes of buffer. This conjugate was centrifuged at 20,000 rpm for 20 minutes in an Beckman model J2-21 using J20 rotor. The conjugate was filtered through a 0.22 µ sterile millipore filter, and a sterile equivolume of glycerol was added to the conjugate which was stored at 4° c.

5.2 Determination of Conjugate Activity

After coupling the conjugates were tested for their activities by using the direct ELISA method. This

was done as follows :

A. Human IgA was used to coat to the polystyrene microtiter plate (5 µg/ml,200 µl). The plates were incubated for 3 hours at 37° c, then washed 3 times with PBS-Tween.

B. 200 ul of serial dilutions of the conjuate in enzyme diluent were added to the wells. The plates were incubated at 37° c 3 hours. They were then washed before adding the enzyme substrate.

5.3 ELISA Method

5.3.1 Indirect ELISA Technique (112) The technique that was used in this thesis is the indirect ELISA technique. IT is the suitable technique for detection antibody such as IgA in serum, saliva, stool extract and intestinal fluids. The steps of the assay are described in detail below :

1. Flat bottomed polystyrene microtiter plates were coated with Ty2 crude lipopolysaccharide (CLPS) 200 µl (5 µg/ml) and incubated at 25° c, overnight. The plates were then washed 3 times with PBS-Tween.

2. Serum, stool extract, saliva, or intestinal lavage samples were serially diluted, (two-fold dilution in PBS-Tween) and 200 µl were added to the corresponding test sample wells and to their corresponding control wells.The plates were incubated at 37°c for 2 hours,

and after the incubation,the plates were washed 3 times with PBS-Tween.

3. The enzyme-antibody conjugate was added (200 µl of a given working dilution) to the wells and incubated for 3 hours at 37° c. In this study the rabbit anti-human α-chain alkaline phosphatase conjugate 1:500 was used and the rabbit anti-human- α-chain urease conjugate was 1:100. The conjugates were added only to the test wells and not to the sample control.

4. After 3 hours of incubation the plates were washed 3 times, and then the enzyme activity was measured by adding p-nitrophenyl phosphate (200 µl of 1 mg/ml) for alkaline phosphatase and the urea-bromocresol purpul (200 µl) for urease to every wells. All were then incubated at 37° c for 30 minutes.

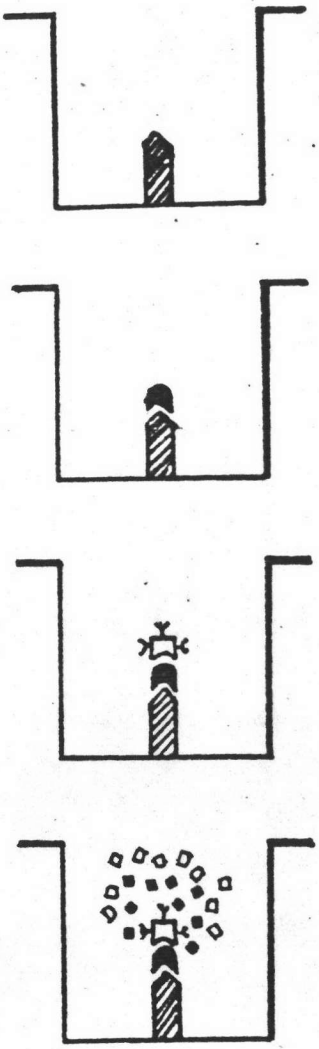
5. Substrate and conjugate controls were carried out in addition to sample controls.

Sample Control = CLPS + test sample + substrate

Conjugate Control = CLPS + conjugate + substrate

Substrate Control (blank) = CLPS + substrate

The controls were carries out under the same condition as the test samples.



CLPS adsorbed to plate

wash

Add sample attaches to CLPS

wash

Add enzyme labelled

rabbit anti human-α-chain

attaches to antibody

wash

Add substrste

Amount Hydrolysis = Amount antibody

Figure 7 Diagram Method for microtitre plate ELISA for the detection and measurement of IgA anti-S.typhi CLPS antigen.