CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Experimental Animals

1.1 Mice

Female Balb/cJ mice 8 to 12 weeks of age were kindly supplied by Department of Veterinary Medicine , AFRIM , Bangkok and used througout these studies . Five to six mice were kept in each cage , and the mice were given unlimited supplied of food and water in an isolation unit . They were kept in an air - conditioned room .

1.2 Rabbits

Normal adults white rabbits were kindly supplied by Animal House , Department of Physiology , Faculty of Medicine , Chulalongkorn University , Bangkok .

2. Strains of Salmonella typhimurium

2.1 Description of Strains

In these studies there were 2 strains of S. typhimurium that to be used as follows :

2.1.1 The virulent S. typhimurium C_5 (serotype 1,4, 5,12) was kindly supplied by Department of Microbiology and Immunology ,University of Adelaide , Australia .

2.1.2 S. typhimurium G_{30} (serotype 1,4,5,12 when grown with galactose) was kindly received from Dr. R. Germanier of Swiss Serum and Vaccine Institute , Berne , Switzerland .

S. typhimurium G_{30} is a gal E mutant (uridine diphosphate (UDP) - galactose - 4 - epimerase - negative) of S. typhimurium C_5 .

2.2 Strain Maintenance

S. typhimurium C_5 was stored as lyophilized cultures at 4° C. For routine use the lyophilized cultures was grown on nutrient agar plates (DIFCO Laboratories , Detroit , Michigan , USA). A single colony was transferred and kept in soft agar stock bottles at 4° C .

S. typhimurium G_{30} was also kept in soft agar stock bottles at 4° C .

2.3 Strain Propagation

Cultures transferred from soft agar stock bottles were grown on nutrient agar plates and a single colony was transferred to Brain heart infusion broth (BBL ,Cockeysville , MD .) at 37°C for 18 hr . These cultures were diluted 1:10 with fresh Brain heart infusion broth and incubated as a shaking culture for 3 hr . This method yielded organisms that were in the log phase . The number of bacteria present was calibrated by optical density at wavelength of 650 nm and confirmed by viable counts . The ultimate number of bacteria in such cultures was about 2×10^9 organisms/ml .

Methods

1. **LD_{50} Determination**

Groups of eight mice were fed orally with Ø.2 ml amounts of 1Ø fold dilutions of S. typhimurium C_s following 5Ø % sat $NaHCO_3$. The bacterial suspensions were made in NSS .

The number of bacteria present was calibrated by optical density at wave length of 65Ø nm and confirmed by viable counts . Death were recorded dialy for up to 3Ø days . LD_{50} values were calculated by the method of Reed and Muench (AppendixI)(144) .

2. **Oral Immunization** (7)

Following feeding with Ø.2 ml of a 5Ø % sodium bicarbonate in NSS , mice were orally immunized with the required dose of organisms , Ø.2 ml of suspension . At this strength of sodium bicarbonate , the stomach acid was neutralized . The oral dose was administered to the mice by means of a syringe and blunt - tipped hypodermic needle (19 g).

3. **Mouse Protection Test** (7)

3.1 **Immunization**

Mice were divided into two groups . The first group of mice was given one dose orally with $1x10^{10}$ live organisms of S.typhimurium G_{30} and the other group of mice was given three doses orally with $1x10^{10}$ live organisms of S.typhimurium G_{30} on each of three alternate days . Each group contained 5Ø - 6Ø mice , 7 and 21

days after the immunization 10 -15 mice were killed to use in the intracellular killing assay , the other 15 - 20 mice were used in the protection test .

3.2 Protection Test

Protection against S.typhimurium C_5 infection (mouse typhoid) was studied by challenging two groups of immunized mice with lethal doses of S.typhimurium C_5 organisms (approximately 1000 LD_{50}). On day 7 , and 21 after oral immunization , 15 - 20 mice from each group were fed orally with $5x10^6$ S. typhimurium C_5 . The number of deaths were recorded for 30 days after the challenge . The control mice received on the same day as the test mice were kept in the animal house and given food and drinking water prior to challenge on days 7 and 21 .

4. Immune Serum (145)

Rabbits were immunized by subcutaneous injections at two sites of 10^8 heat - killed (90 min , 60° C)S.typhimurium C_5 suspended in NSS and mixed in equal parts with Freund' s complete adjuvant .

Each rabbit received booster injections of 10^8 heat - killed bacteria every week for 4 weeks .

After this period ,blood was obtained by puncture of the ear vein and was clotted at room temperature ; the serum was collected , and the pressure of antibodies against S.typhimurium C_5 was checked by a bacterial agglutination test..

Serum was stored in small aliquots at -20° C . Inactivation of the serum was achieved by heating for 30 min at 56° C .

Preliminary experiments revealed that sera from normal

rabbits lacked antibodies against S.typhimurium C_5 .

5. Bacterial Agglutination Test (146)

5.1 Preparation of the Antigen for the agglutination test

S. typhimurium C_5 used for antigen preparation was grown on nutrient agar plates (DIFCO Laboratories , Detroit , Michegan , USA) at 37° C for 18 hr , and a single colony was tranferred to Brain heart infusion broth (BBL Microbiology Systems , Becton Dickinson and CO , MD) at 37° C for 18 hr . These cultures were diluted 1:10 with fersh Brain heart infusion broth (BBL Microbiology Systems , Becton Dickinson and CO , MD) and incubated as a shaking culture for 3 hours . The cultures were harvested by centrifugation at 1,500 g for 10 min , and washed twice with NSS . The suspension was diluted to the desired concentration in NSS . The number of bacteria present was calibrated by optical density at wave length of 650 nm and confirmed by viable counts . Heat at 60° C for 90 min . When this antigen was to be stored at 4° C,added 1/10,000 merthiolate .

5.2 Standardization of Salmonella typhimurium C_5 Antigen Concentration for the Agglutination Test

The suspension of S.typhimurium C_5 antigen was diluted into three dilution containing 10^{10} , 10^9 , 10^8 organisms/ml , respectively . Each dilution of antigen was tested with serial dilutions of antiserum to determine the optimal dilution of antigen which gave the highest agglutination titer .

In this study , the dilution of 10^9 organisms/ml was

used for the working antigen .

5.3 The Bacterial Agglutination Method

The agglutination was performed in small test - tubes 75 x 9 mm .The sera were inactivated at 56° C for 30 min before use .

Doubling dilution of antiserum were prepared in eight tubes as follows :

Tube No .	1	2	3	4	5	6	7	8	9
Dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	0

0.5 ml of antigen (10^9 organisms/ml) was added to each tube . The last tube containing no serum , was set up as a negative control . Final dilutions were then :

Tube No .	1	2	3	4	5	6	7	8	9
Dilution	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	0

The contents of each tube was mixed thoroughly and incubated for 2 hr in a 37° C water bath before standing overnight in refrigerator(4° C).

Positive agglutination was seen as diffuse clumping, whereas a negative reaction appeared as a small button that had settled to the bottom of the tube .

The titer of the antiserum was given as the reciprocal of the highest dilution that caused clumping .

6. Peyer' s Patches Cell Cultures

PP Cells were collected from the small intestines of mice immunized orally by the following regimens :

- Mice fed orally 7 days earlier with $1x10^{10}$ S. typhimurium G_{30} .

- Mice fed orally 21 days earlier with $1x10^{10}$ S. typhumurium G_{30} .

- Mice fed orally with $1x10^{10}$ S.typhimurium G_{30} on each of three alternate days and PP were removed on day 13 (7 days after the last oral feeding).

- Mice fed orally with $1x10^{10}$ S.typhimurium G_{30} on each of three altertnate days and PP were removed on day 27 (21 days after the last oral feeding).

The controls (non - immunized mice) were also set up .

6.1 Removal of Peyer' s Patches from Mice (147)

Mice were sacrificed by cervical dislocation . The small intestines were removed , seperated and freed from surrounding mesentery . Ten ml NSS was pass through each intestine to get rid of the intestinal contents and then rinsed with NSS . PP were identified , the serosal side of each patch was carefully dissected and isolated PP were then placed into Incomplete medium An arerage of 8 PP per animal was obtained , although variation in the number and size of PP occurred from animal to animal .

6.2 Extraction of Peyer' s Patch Cells by Enzymatic Treatment (147)

The PP from 8 to 10 animal were placed in a petri dish with 20 ml of enzyme solution and incubated at 37° C for 30 - 45 min . The medium containing dissociated cells was collected by aspiration and 20 ml of fresh enzyme solution was immediately added to the remaining tissue which was incubated again for 30 - 45 min . This procedure was repeated 3 - 4 times .

6.3 Identification of macrophages

6.3.1 Nonspecific Esterase Staining (148)

6.3.1.1 The smear was fixed in fixative agent for 30 sec at 4° C .

6.3.1.2 Washed with water and air dried .

6.3.1.3 The slide was immersed in freshly prepared incubation medium for 45 min at 37° C .

6.3.1.4 The smear was washed with water and counterstained with 1 % methyl green for 1 to 2 min .

6.3.1.5 Washed with water , dried mount with permount , and examined with a light microscope .

Esterase activity identifed by the presence of granules , mainly in the cytoplasm of macrophages was seen as dark red color .

Esterase - positive Cells with morphological characteristic compatible with macrophages were enumerated in PP preparations by counting the positive cells per 1000 total cells visualized with a light microscope .

6.3.2 Latex Ingestion Test (149)

6.3.2.1 Cells were suspended at 10^5 /ml in complete medium and incubated for 2 hr at 37° C with 1:50 dilution of 10 % latex particles (Bacto latex 0.81 um , DIFCO , Detroit , Michigan , USA) .

6.3.2.2 Cells were centrifuged through 5 % bovine serum albumin (CSL , Melbourne , Australia) to remove free particles .

6.3.2.3 Cells that contained greater than five latex particles were scored as positive .

7. Intracellular Killing Assay

7.1 Preparation of Peyer's Patch Cells for Intracellular Killing Assay . (145)

The medium containing dissociated cells was placed into a 50 ml sterile centrifuge tube containing 20 ml of complete medium . Cells were pelleted at 450 x G for 10 min at room temperature , washed twice in complete medium . Cells were counted and viable cells enumerated in 0.4 % trypan blue . The cells were examined within 2 min in a microscope counting - chamber . The proportion of cells that had taken up the dye was determined by a

survey of at least 100 cells . Viable cells were those that did not take up the dye .

7.2 Preopsonization of Salmonella typhimurium C_s- (145)

Microorganisms were preopsonized by incubation of 10^7 bacteria /ml with the desired concentration of inactivated rabbit immune serum for 30 min at 37° C in a shaking water bath , followed by centrifugation for 10 min at 1500 x G . The bacteria were washed twice with NSS and suspended in gelatin - HBSS to a concentration of 10^7 /ml . The presence of immunoglobulins on the surface of the bacteria was checked by immunofluorescence after incubation of preopsonized with bacteria fluorescein - labeled swine anti - rabbit immunoglobulin .

7.3 Indirect Immunofluorescence Antibody Test (150)

7.3.1 Preparation of Conjugate Dilutions

The FITC conjugated anti - rabbit immunoglobulins was serially two fold diluted in PBS 7.2 pH begining with the dilution of 1:10 and going beyond the range of activity .

7.3.2 Titration of the Conjugate .

The fluorescein isothiocyanate (FITC) conjugated anti - rabbit immunoglobulins was assayed with preopsonized S. typhimurium C_s to determine the optimal dilution for use prior to performing the test . The serial two - fold dilutions of conjugate were then reacted with preopsonized S. typhimurium C_s .

The optimal dilution of the Conjugate was

determined as the highest dilution producing 3^+ to 4^+ fluorescence of preopsonized S. typhimurium C_s and no staining of an unpreopsonized S. typhimurium C_s control .

7.3.3 **Setting Up the Indirect Immunofluorescence Antibody Test** .

The FITC conjugated anti - rabbit immunoglobulins was diluted in PBS 7.2 pH to make the dilution of 1:80 , as the optimal dilution of conjugate for use . The IFA - test was performed as follows :

7.3.3.1 Preopsonized S. typhimurium C_s was spotted onto slides , which were air - dried at room temperature prior to fixation for 10 min in cold methanol .

7.3.3.2 The air - dried smears were then overlaid with 20 ml of 1:80 dilution of FITC conjugated anti - rabbit immunoglobulins and allowed to stand in a moist chamber at room temperature for 30 min .

7.3.3.3 After this incubation period the conjugate dilution was rinsed off with PBS 7.2 pH and then washed in three changes of PBS 7.2 pH for 10 min at each change .

7.3.3.4 The air - dried smears were then mounted with PBS - buffered glyceral solution and examined under a coverslip with fluorescent microscope .

A typical fluorescence indicated preopsonization of S. typhimurium C_s while no fluorescence was seem

with unpreopsonized cells .

7.4 In Vitro Intracellular Killing Assay (145)

The ability of macrophages to kill challenging S. typhimurium C_5 in vitro was examined as follows :

7.4.1 PP cell suspensions containing 5 x 10^6 macrophages and 5 x 10^6 preopsonized bacteria/ml or 5 x 10^6 bacteria/ml were incubated at 37° C in a shaking water bath for 20 min .

7.4.2 After 20 min of incubation , phagocytosis was terminated by shaking the tube in crushed ice at 4° C for 1 min .

7.4.3 The noningested bacteria were removed by centrifuging the PP cell suspension for 6 min at 75 x G (Centrifuge Moded DPR - 6000 , DAMON/IEC Division) and washing three times with 1 ml ice - cold gelatin - HBSS .

7.4.4 The macrophages containing ingested bacteria were reincubated at a concentration of 5 x 10^5 cells/ml in the presence or absence of desired concentration of inactivated rabbit immune serum at 37° C in shaking water bath .

7.4.5 At various intervals (0 , 30 , 60 , and 90 min) , 50 ul samples of this suspension were added to 450 ul distilled water containing 0 .01 % (w/v) bovine serum albumin , and these were mix vigorously to lyse the macrophages .

7.4.6 The number of viable bacteria was then determined microbiologically . Serial ten - fold dilutions in NSS

were made from the sample and aliquots of Ø.1 ml of the dilution were pipetted on to each of two Tetrazolium - galactose agar plates and immediately spread . The plates were incubated at 37° C for 24 hr , and the colonies were counted . The number of viable bacteria/ml was calculated from the means of the colony counts of duplicate plates .

7.5 Determination of the optimal Concentration of Immune Serum for In Vitro Intracellular Killing Assay

To determine the optimal concentration of immune serum for in vitro intracellular killing assay , $5x10^6$ macrophages/ml were incubated with $5x10^6$ preopsonized S.typhimurium C_5 in the presence of various concentrations of immune serum (1 , 5 , 7 , 1Ø , 15 and 2Ø % v/v) at 37° C .

That concentration of immune serum which gave the maximum in vitro intracellular killing of S.typhimurium C_5 was regarded as the optimal concentration .

All values represent the mean of three experiments . Intracellular Killing at a given time - point is expressed as the percentage decrease of the initial number of viable intracellular bacteria according to the following formula : $K(t) = (1 - Nt/No) \times 100$, in which K (t) is the intracellular Killing index at time t, No is the number of viable intracellular bacteria at time 0 , and Nt is the mean number of viable intracellular bacteria at time t . Statistical analysis was performed with Student ' s t test .