Chapter III

MATERIAL AND METHODS
I. Maintenance of Laboratory Infections:

A. Strain: The strains of *Plasmodium berghei* and *Plasmodium gallinaceum* used in the present investigation were obtained in 1975 and 1976 respectively from National Institute of Communicable Diseases (NICD) through the courtesy of the Director of the Institute. The *P. berghei* strain was received at NICD in 1949 from Professor Emile Brumpt in Paris and has since been maintained by serial blood passages in mice. The *P. gallinaceum* strain obtained at NICD around the same time, was being maintained in white leghorn fowls by blood passages interrupted by occasional cyclic passage. Since the acquisition of the two strains in our laboratory *P. berghei* has been maintained by weekly syringe passage in Swiss mice or albino rats and *P. gallinaceum* in leghorn chicks by blood passage. For serial passage blood from one or two animals showing high parasitaemia is pooled by cardiac puncture and after counting the parasites, it is diluted with the appropriate quantity of the anticoagulant. The diluted inoculum is then injected intra-peritoneal into the rodent hosts and intramuscular into the chicks. The anticoagulant is prepared by dissolving 2 gm of tri-Sodium Citrate and 0.85mg Sodium chloride in 100 ml distilled water and autoclaving the solution at 15 lbs pressure for 15 minutes.

B. Host: Laboratory bred strains of Swiss mice, albino rats (Druckrey strain), *Mastomys natalensis* and Golden hamsters have been employed in various experiments with *P. berghei*. Healthy colonies of these rodents are being maintained under standard temperature conditions in the 'Animal House' wing of the Institute. A day old white leg horn chicks were purchased from the local Government Poultry Farm for maintenance of *P. gallinaceum* infection. These chicks were kept in the 'Animal House' for 7-10 days for acclimatization before being used in various experiments.
C. **Vector**: Mosquitoes of the species *Aedes aegypti* obtained from the colonies maintained in the laboratories for a number of years have been used for the cyclic transmission of *P. gallinaceum* in chicks.

D. **Rearing of the mosquitoes**: The colonies of *Aedes aegypti* were kept in insectary under controlled environmental conditions. The temperature was thermoregulated between 76-80°F and the relative humidity varied from 70-80%. The four larval generations from the first instar to the fourth instar, kept in enamel bowels, were fed on powder yeast. The pupae when formed were pipetted into plastic petri-dishes and kept for hatching in the mosquito-net cages. Sucrose solution in cotton swabs was kept in each cage for survival of the mosquitoes. Mosquitoes were starved for a day before feeding on the chicks.

E. **Cryo-preservation of the parasites**: Plastic vials of 2 ml capacity were used for freeze preservation of samples of the strains of *P. berghei* and *P. gallinaceum* by method of Jeffery (1962). The preservative was prepared by mixing 20 ml glycerol in 80 ml phosphate buffer (pH 7.2). 1 ml. of this solution was added to each vial and the vials were autoclaved for 15 minutes at 15 lb. pressure. For preservation 1 ml. of fresh citrated blood was added to each vial thus giving a final glycerol concentration of 10%. The vials were slowly immersed in the liquid air (-170°C) container. In order to produce infection with frozen parasites, the vials were slowly thawed to 37°C and the contents injected into two or three animals.

F. **Examination of blood smears**: For recording the course of infection, thin blood films were made from a drop of blood obtained after pricking the tail vein in rodent hosts and the wing vein in chicks. The slides were stained for a minute with Wright's stain and washed with phosphate buffer (pH 7.2). After drying, the blood smears were observed under the oil-immersion objective of a
binocular microscope. The percentage parasitaemia was recorded from the ratio of parasitized red cells to normal red cells in 50 microscopic fields. A minimum of 50 fields were checked before labelling a smear as negative.

G. Preparation of stain and staining buffer: The working solution of Wright's stain was prepared by mixing the following ingredients.

- Powder Wright's stain (L. Merck) - 2.5 gm.
- 100% pure methanol - 970 ml.
- Glycerol - 30 ml.

This mixture was kept for ripening for a week to ten days before being put into use for staining the slides.

The two stock solutions for the phosphate buffer were prepared as given below:

- \( S_1 \): 9.50 gms. \( \text{Na}_2\text{HPO}_4 \) in 1000 ml distilled water.
- \( S_{II} \): 9.07 gms. of \( \text{KH}_2\text{PO}_4 \) in 1000 ml. distilled water.

For preparing the working solution, 72 ml of \( S_1 \) and 28 ml of \( S_{II} \) were added to 900 ml. distilled water and pH of the resulting solution was adjusted to 7.2.

II. Drugs and Antibiotics

A. Drugs: Commercial samples of the following antimalarial drugs have been used in various chemotherapeutic evaluations. Chloroquine (Hesochrin), Bayer (India) Limited, Bombay; Amodiaquine (Comoquin), Parke-Davis (India) Limited, Bombay; Mepacrine, British Pharmaceutical Labs., Bombay; Pyrimethamine (Daraprim), Burroughs Wellcome & Co., (I) Pvt. Ltd., Bombay; Diamino-diphenyl sulfone (DDS, Lapsone), Bengal Immunity Co., Ltd., Calcutta; Sulphadiazine, May & Baker (India) Pvt. Ltd., Bombay and Sulphanilamide, Cyper Pharma, New Delhi. Pure sample of Primaquine diphosphate used in this study was synthesized in the Medicinal Chemistry Division of the Institute. Pure sample of Mefloquine hydrochloride (H021-5998/001, lot 18/64253)
was received as a gift through the courtesy of Messrs Roche Products Ltd., Basel.

B. Antibiotics: Commercial samples of the following antibiotics were used for antimalarial evaluation against *P.berghei* and *P.gallinaceum*. Doxycycline hydrochloride (Biodoxi), Biochem Pharmaceuticals Industries, Bombay; Demeclocycline hydrochloride (Ledemycin), Cynamide India, Ltd., Bulsar; Chlortetracycline hydrochloride (Aureomycin), Cynamide India Ltd., Bulsar; Tetracycline hydrochloride (Tetracycline), Indian Drugs and Pharmaceutical Limited, Hyderabad; Oxytetracycline hydrochloride (Terramycin), Pfizer (India) Ltd., Bombay; Erythromycin stearate (Erythrocin), Abbot Laboratories (India) Pvt. Ltd., Bombay; Chloramphenicol (Chloromycetin), Parke-Davis (India) Ltd., Bombay, and pure sample of minocycline hydrochloride (Minocin), American Cyanamide Company, New York.

C. Administration of drugs and antibiotics: Various antimalarial drugs and antibiotics were dissolved in appropriate quantity of distilled water to have the required amount in 0.5 ml liquid. The aqueous solution/suspension was administered orally.

III. Experimental Studies:

A. Selection of host: The course of infection of the normal (sensitive) strain of *P.berghei* was compared in Swiss mice (18-20 gm), albino rats (20-25 gm), *Mastomys natalensis* (25-30 gm) and Golden hamsters (30-40 gm). Each animal was inoculated intraperitoneally with 10 million parasitized red cells per 20 gm. wt. of the animal. The day of infection has been referred to as Day zero, (D 0), the subsequent days being *D*+1, *D*+2 and so on. The percentage parasitaemia was recorded everyday till the death of the animal or till there was no patent infection in blood smears for 4-6 days. In the second experiment, groups of albino rats of 25 gm. wt., 50 gm. wt., 100 gm. wt. and 150 gm wt. were inoculated with 10 million infected red cells per
25 gm animal on day zero and the course of parasitaemia was recorded till day +21 or till death. In the third experiment, 25 gm wt. rats were inoculated with different concentrations of the infected red cells varying from $0.62 \times 10^6$ to $100 \times 10^6$ parasites. The course of parasitaemia and the day of death were recorded as above.

B. Chemotherapeutic studies:

1. Blood schizontocidal activity: To determine the chemotherapeutic response to P. berghei infection, each antimalarial drug and antibiotic was administered at 4 dose levels to a group of 6-10 animals per dose level. The activity was determined on the basis of (i) mean survival time (ii) minimum effective dose and (iii) percent suppression of parasitaemia in treated from the untreated group.

   (i) Determination of mean survival time: Groups of 10 mice at four dose levels for each antimalarial drug, were inoculated on day zero with 10 million parasites. Similarly, groups of 6 mice at four dose levels for each antibiotic were inoculated on day zero with 25 million parasitized cells. The drugs were administered from day 0 to day +3 and the day of death of each animal was recorded till the observation period of day +21 post inoculation. The arithmetical average for each group was recorded as the mean survival time.

   (ii) Determination of minimum effective dose: The minimum effective dose of antimalarial drugs to sensitive strain of P. berghei was determined in four rodent hosts. Groups of 8 animals each of Swiss mice (20 gm), albino rat (25 gm) Mastomys (30 gm) and Hamsters (35 gm) were inoculated on day zero with 10 million parasitized cells per 20 gm animal. Four dose levels of each drug were administered once daily from day 0 to day +3, starting four hours after inoculation of parasites. Blood smears from all animals were examined from day +4 to day +7 to determine the erythrocyte infection rate (EIR). The lowest dose capable of
suppressing the parasites till day 7 was recorded as the minimum effective dose.

(iii) Determination of $ED_{50}$ and $ED_{90}$ values: The effective doses showing suppression of 50% ($ED_{50}$) and 90% ($ED_{90}$) population of the parasites after the drug treatment were determined for various drugs and antibiotics in Swiss mice. Four dose levels of each drug at 2 fold increasing dilution and of each antibiotic at 3 fold increasing dilution, were used. Six mice at each dose level were infected with 25 million parasitized red cells. The drug treatment was given from day 0-day +3; once daily for the antimalarial drugs and in two divided doses daily for the antibiotics. Erythrocyte infection rate (EIR) on day 4 was recorded for each animal and the same was compared with the corresponding value of the untreated group to get percentage suppression.

$$\text{Percent suppression} = \left( \frac{\text{EIR in treated group}}{\text{EIR in untreated control}} \right) \times 100$$

$ED_{50}$ and $ED_{90}$ values were determined by plotting a graph representing percent suppression of parasitaemia against the corresponding dose in the log scale.

2. Tissue schizontocidal activity: The tissue schizontocidal activity of some of the antimalarial drugs and antibiotics was determined in chick- $P.gallinaceum$ - $A.aegypti$ model. For infecting the mosquitoes, 4-5 day old mosquitoes were fed on infected chick showing 40-60% parasitaemia. The chick was anaesthetized by intramuscular injection of 'Nembutal' (40 mg/kg) and placed over mosquito cage, containing 300-400 prestarved mosquitoes, for sufficient period to enable most of the female mosquitoes to have blood meal. During the subsequent days, mosquitoes were fed on sucrose solution. Sample dissections were made from day 4 to day 7 to check for presence of the oocysts. For transmission of infection to normal chicks, 5-7 female infected mosquitoes were transferred to smaller cages and allowed to feed on the healthy chick.
To assess the prophylactic activity of a drug, randomly infected chicks were pooled in batches of six. Each drug was administered for 6 consecutive days in two divided daily doses from day 0 to day +5 to each group of chicks. The first dose was given on day 0, a few hours after sporozoite transmission. Blood smears of all the chicks were checked from day 6 onwards till death or till day 21 post-infection.

Similarly prophylactic activity of a number of antibiotics was determined. Each antibiotic was administered at four dose levels of three fold increasing dilution to a batch of six chicks per dose level. After 21 days, the representatives from surviving groups were reinfected with fresh batch of mosquitoes to check the susceptibility of the host. Subinoculation test was also carried out on day +21 from the surviving batch to detect latent infection if any. For this cardiac blood was inoculated into healthy chicks in ratio of 1:1 and the latter were checked for blood infection for 14 days.

3. Activity after single dose treatment: Antimalarial drugs were administered orally to a group of 30-40 Swiss mice at two dose levels for each drug. Mice in batches of six to eight from each level were subsequently challenged at regular intervals with 10 million parasitized red cells. The course of infection in the treated batches was compared with the untreated controls and all the mice were observed till death or till day 21 post infection.

IV. Studies on Drug Resistance

A. Selection of resistant strains: Four strains of P.berghei resistant to pyrimethamine, chloroquine, primaquine and mefloquine were selected in weanling rats (16-20 gm) by the method of interrupted subcurative therapy (Hollo, 1952). The term implies that the parasites were treated with subcurative doses of drug, interruptedly, depending on the
rate of increase in parasitaemia, thereby subjecting the parasite population to selection pressure at frequent intervals. The level of resistance was checked in the weanling rats as well as in other rodent hosts by the standard four day test. In this test the response of the drug treated group was compared with the untreated controls after drug treatment from day 0 to day +3.

B. Stability of resistance: The stability of the resistance was checked (i) after serial passages of the resistant parasites in weanling rats in the absence of drug for prolonged periods and (ii) after the cryopreservation of samples of resistant strains in liquid air. In both cases the level of resistance was checked by treating groups of rats for four doses from day 0 to day +3 at various dose levels and the fate of infection was followed till death of the host.

C. Sensitivity of the resistant strains to other antimalarial drugs and antibiotics: The cross-sensitivity of the resistant strains to other antimalarials was determined in rats and mice on the basis of minimum effective dose (MED). The MED was determined, as for the sensitive strain, by the four day treatment (Day 0-3) at various dose levels and the degree of infection was checked from day +4 to day +7.

The \( \text{BD}_{50} \) and \( \text{BD}_{90} \) values of various antimalarials and antibiotics were determined in mice for pyrimethamine, chloroquine and primaquine resistant strains in the same way, as for the parent normal strain. The index of resistance (AIR) was determined by comparing the corresponding \( \text{BD}_{50} \) and \( \text{BD}_{90} \) values of sensitive and resistant strains.

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\text{Index of resistance} = \frac{\text{ED}_{50}/\text{ED}_{90} \text{ of resistant strain}}{\text{ED}_{50}/\text{ED}_{90} \text{ of sensitive strain}}.
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