INDIAN JOURNAL OF MALARIOLOGY

Volume 19 Number 2

December 1982

MALARIA RESEARCH CENTRE
Indian Council of Medical Research
22, Sham Nath Marg,
Delhi-110 054

INDIAN JOURNAL OF MALARIOLOGY

HALF YEARLY

SUBSCRIPTION RATE

Annual subscription	India	Rs. 30.00*
	Other countries (including airmail postage)	$ 10.00

*50% Discount would be admissible to the individual subscriber on annual basis.

Payments in respect of subscription may be sent by money order/bank draft/postal order crossed and marked payable to Malaria Research Centre, 22, Sham Nath Marg, Delhi-110 054.

INDIAN JOURNAL OF MALARIOLOGY

INDIAN JOURNAL OF MALARIOLOGY

CONTENTS

Volume 19
Number 2
December 1982

(contd.)

Short Notes

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 73-82

Proteinases of *Plasmodium knowlesi*

H.S. BANYAL[1], V.C. PANDEY[2] and G.P. DUTTA

Cell-free parasites obtained from schizont infected cells by saponin lysis possessed 3-5 times more acid and alkaline proteinase activities as compared to the ghosts (erythrocyte membranes), and 30-50 times more activity than that of the hemolysate. Further, a comparison of proteolytic activity of three stages, namely: merozoites, rings and schizonts showed that both acid and alkaline proteinase activity was 2-3 fold higher in merozoites as compared to the other stages. The proteinases of cell-free parasites showed two pH optima (4.0 and 7.0) and higher affinity towards denatured haemoglobin as compared to crystalline haemoglobin and also effectively hydrolysed haemoglobins from several other sources such as human, rhesus monkey, assamese monkey, rabbit, rat, hamster, mouse and guinea-pig. Parasite acid proteinase was effectively inhibited by Zn^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Fe^{3+}, sodium diethyldithiocarbamate and pepstatin, and alkaline proteinase by Zn^{2+}, Soybean trypsin inhibitor and phosphoramidon. Primaquine had slight inhibitory action on acid proteinase and oxytetracycline on alkaline proteinase.

INTRODUCTION

Proteolytic enzymes of several species of plasmodia including *P.falciparum* (Levy *et al.*, 1974), *P.knowlesi* (Cook *et al.*, 1961; Cook *et al.*, 1969; Levy *et al.*, 1974), *P.berghei* (Cook *et al.*, 1961; Levy and Chou, 1973; 1974) and *P. gallinaceum* (Moulder and Evans, 1946) have been demonstrated. Cook *et al.* (1961) reported two different proteinases in *P.knowlesi* which were maximally active at pH 5.0 and 8.0, while in *P.berghei* they were active at pH 4.0 and 8.0. These proteinases were inhibited by diisopropylphosphorofluoride but were unaffected by thio-group inhibitors. Levy and Chou (1973) reported maximum activity of proteinase of *P.berghei* at pH 2.5-3.0, while Levy *et al.* (1974) showed maximum activity at pH 3.6 in *P.falciparum* and *P.knowlesi* but they could not detect any activity at alkaline pH. In view of the conflicting reports in literature on the pH optima of proteinases of *P.knowlesi* and since detailed characteristics of these enzymes including stability, hydrolysis of different substrates, the effect of metal ions, metabolic inhibitors/activators, drugs and antibiotics had not been studied earlier, a detailed study on the proteolytic enzymes of *P.knowlesi* has been carried out and results are presented in this communication.

Accepted for publication, 22 June 1982.

Division of Microbiology[1] & Biochemistry[2]
Central Drug Research Institute, Lucknow

MATERIAL AND METHODS

Adult rhesus monkeys *Macaca mulatta* of either sex, weighing about 3-6 kg were kept under 12 hr photoperiodicity with fluorescent lights on from 7.00 hr to 19.00 hr. A strain of *P.knowlesi* (W_1) kindly donated by Professor P.C.C. Garnham was used in this study.

Merozoites— Blood from *P.knowlesi* infected monkey was collected in acid citrate dextrose (ACD) (Kessel *et al.* 1965) by cardiac puncture when the parasitaemia was 15-40% and the parasites were mostly multinucleated schizonts. It was centrifuged at 800g for 10 minutes. Plasma and buffy coat were removed and the schizont-infected cells, which form a brownish layer, were separated from the underlying erythrocytes. They were washed thrice with medium 199. 0.05 ml of packed cells were cultured in presence of 3.0 ml medium 199 and 0.3 ml normal inactivated rhesus monkey serum in a 50 ml Erlenmeyer culture flask. The flasks were then kept at 37° C under an atmosphere of 7% CO_2, 1% O_2 and 92% N_2. After 5-6 hrs incubation, when most of the schizonts had rup-

urea), the cell suspension was centrifuged at 750g for 15 minutes in a refrigerated centrifuge. The supernatant was again centrifuged at 2,000g for 20 minutes and merozoites were collected in the sediment and then washed thrice with prechilled normal saline and stored at -20° C until used.

Cell-free parasites, ghosts and hemolysate— The infected erythrocytes were harvested as mentioned above and were lysed for 30 minutes at 4° C with 0.2% (w/v) saponin in saline. The lysed suspension was then centrifuged at 7,000 g for 30 minutes which yielded three fractions: The uppermost layer or hemolysate was followed by ghosts (red cell membranes) and the parasites were present in the sediment. The parasites were separated from hemolysate and ghosts by repeated washings with cold normal saline by centrifugation at 7,000g for 30 minutes and designated as cell-free parasites. Erythrocytes of normal monkey were also processed like the parasitized erythrocytes and served as control.

Preparation of homogenate.— Freshly harvested erythrocytes, ghosts, cell-free parasites and merozoites were homogenized in Potter-Elvehjam homogenizer at 4° C in prechilled glass distilled water. The resulting homogenate was centrifuged at 1,800g for 15 minutes and used as crude enzyme.

Assay of proteinase. Proteinase activity was determined according to a slightly modified method of Anson (1938). The assay mixture contained 100 μ moles of buffer (for acid proteinase Walpole buffer, pH 5.0 and phosphate buffer pH 7.2 for alkaline proteinase), 70 μ moles of cysteine hydrochloride (adjusted to required pH with NaOH), 0.05% (w/v) haemoglobin and 0.3 ml suitably diluted enzyme in a final volume of 2.0 ml. Tubes were incubated at 37° C for 60 minutes with occasional shaking. The reaction was stopped by the addition of 1.0 ml of 10% (w/v) prechilled trichloroacetic acid (TCA) and the precipitate was removed by centrifugation at 1,500g for 15 minutes in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. In controls, the enzyme was supplemented after the addition of TCA. A suitably diluted aliquot of the protein free supernatant was taken for colour development with Folin and Ciocalteau's reagent (Lowry *et al.*, 1951).

Unit of enzyme was that amount which liberated one μ mole of tyrosine in 60 minutes.

Preparation of different haemoglobins-Crystalline or denatured haemoglobin was procured from commercial sources.

Haemoglobin from the blood of man, monkey, rabbit, guinea-pig, hamsters, rats and mice were prepared in the laboratory. 10.0 ml blood was collected in 1.0 ml of 2% (w/v) sodium citrate in 0.85% sodium chloride and centrifuged at 800g for 10 minutes. After removing plasma and buffy coat, the erythrocytes were washed thrice in normal saline and lysed by repeatedly freezing and thawing and then centrifuged at 7,000g for 30 minutes. The supernatant (hemolysate) was boiled for 15 minutes and the precipitate was homogenized. 0.5 ml aliquot was then added to the reaction mixture.

Effect of activators, inhibitors, drugs and antibiotics— The desired concentrations of metal ions, activators, inhibitors, antimalarial drugs and antibiotics were added to the tubes containing suitable enzyme preparations and incubated at 37° C for 10 minutes along with the controls, and then other constituents of assay mixture were added. The tubes were then incubated at 37° C for 30-60 minutes and the enzyme activity was determined.

RESULTS

Initial studies on the proteolytic enzymes of the total homogenate of normal and *P.knowlesi* infected red cells of rhesus monkey showed that infected red cells showed higher activity of proteolytic enzymes as compared to normal red cells (Table 1). Fractions of the infected red cells obtained after saponin lysis namely cell-free parasite, ghost (red cell membranes) and hemolysate when assayed for both acid and alkaline proteinases, showed that most of the activity of these enzymes was localised in the parasite fraction whereas the membranes (ghosts) showed lower activity and the hemolysate showed least activity. Parasite fraction contained nearly 10-12 times more activity of acid proteinase and 7-8 times more activity of

Table 1 Acid and alkaline proteinase activity of normal and *P. knowlesi*-infected rhesus erythrocytes, cell-free parasites and cultured merozoites of *P. knowlesi*

Enzyme	Erythrocytes	Parasite stage	Specific Activity					
			Total	Hemolysate	Ghost	Parasite	Merozoite	
							Without Triton X-100	With Triton X-100 0.05% (v/v)
Acid proteinase	Normal		0.010	0.006	0.020			
	Infected	Ring	0.060	0.020	0.185	0.620		
		Schizont	0.070	0.032	0.170	0.850	Nil	2.16
Alkaline proteinase	Normal		0.023	0.008	0.035			
	Infected	Ring	0.090	0.010	0.190	0.685		
		Schizont	0.102	0.020	0.210	0.775	Nil	1.97

alkaline proteinase as compared to that of total RBC. Specific activity of both the enzymes was higher in the schizonts as compared to the rings. Proteinases of the merozoites could be demonstrated only in the presence of 0.05% Triton X-100 which appears to release membrane bound enzymes. Merozoites preparation showed 2-3 times higher proteinase activity when compared to the cell-free parasites and nearly 20-40 times higher activity compared to the infected red cells (Table 1).

Studies on the pH optima with denatured haemoglobin as substrate showed that the proteolytic enzymes of the cell-free parasites were maximally active at pH 4.0 and 7.0 (Fig. 1) and that of red cell membranes (Ghosts) at pH 4.5 and 7.0 (unpublished). Parasite enzymes were slightly more active at alkaline pH than the acidic pH. With crystalline haemoglobin the acid and alkaline proteinase activity was more or less similar while with denatured haemoglobin the alkaline proteinase activity was definitely higher (Table 2). The data on the hydrolysis of haemoglobin from red cells of different species by parasite proteinases are presented in Table 2. Haemoglobin prepared from red cells of various species was more readily hydrolysed by acid than the alkaline proteinases of parasite. The haemoglobin from the red cells of species refractory to *P.knowlesi* was also hydrolysed by parasite proteases though to a slightly lesser extent than the haemoglobin isolated from human and rhesus monkey red cells which are susceptible to *P.knowlesi* infection.

Stability of parasite acid and alkaline proteinases stored at −20° C for a period upto 30 days was studied (Table 3). The enzymes were found to be stable upto 30 days at −20° C but not at 4° C. Studies on time activity relationship showed that increase of activity was linear upto 60 minutes of incubation. Optimal enzyme activity was obtained between 40° C-50° C in case of acid protease and between 37° C-45° C in the case of alkaline protease. The enzyme became inactive at 100° C (Figs. 2 and 3). Acid proteinase activity increased upto 0.5% concentration of haemoglobin while the activity of alkaline proteinase was maximum with 0.05% hae-

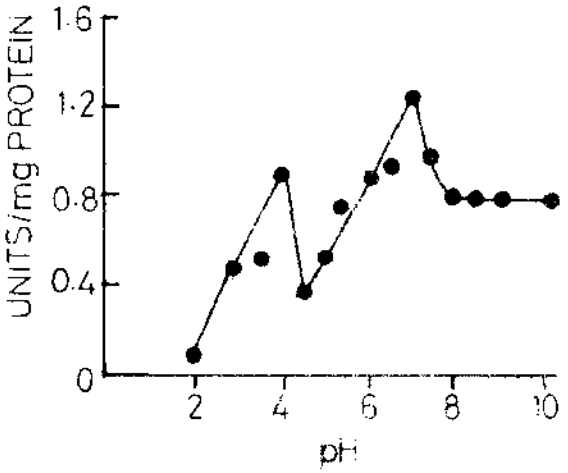


Fig. 1. Proteolytic enzyme activity of cell-free parasite at different pH.

Table 2. Hydrolysis of haemoglobin and casein by acid and alkaline proteinase of *P.knowlesi*.

Substrate		Specific Activity	
		Acid proteinase	Alkaline proteinase
Haemoglobin			
Crystalline	0.05% (w/v)	0.448	0.377
Denatured	2.5%	0.433	0.772
Human		0.757	0.528
Monkey		0.609	0.441
Rabbit		0.577	0.419
Guinea-pig		0.343	0.245
Hamster		0.512	0.362
Rat		0.513	0.369
Mouse		0.476	0.346
Casein	1.5% (w/v)	0.252	0.528

For alkaline proteinase 200 mg of casein was dissolved in 20 ml 0.1M phosphate buffer, pH 7.6. The mixture was boiled for 15 minutes and volume was made upto 20 ml with distilled water. For acid proteinase procedure was same except that 0.1M acetate buffer, pH 4.0 was used. Casein was not completely soluble at pH 4.0 so the solution was centrifuged at 2000 g for 15 minutes and the supernatant was used as substrate.

Preparation of different haemoglobins is given in Material and Methods.

Table 3. Stability of acid and alkaline proteinase of *P.knowlesi* in the cold.

Time (Days)	Acid proteinase			Alkaline proteinase		
	Parasite -20°C	Homogenate -20°C	Homogenate +4°C	Parasite -20°C	Homogenate -20°C	Homogenate +4°C
0	0.355	0.525	0.525	0.140	0.185	0.185
1	0.360	0.526	0.515	0.228	0.230	0.206
2	0.381	0.517	0.510	0.221	0.227	0.208
4	0.395	0.520	0.480	0.230	0.230	0.208
6	0.436	0.518	0.354	0.225	0.252	0.212
15	0.440	0.517	ND	0.230	0.227	ND
30	0.446	0.545	ND	0.262	0.254	ND

ND = Not determined.

Table 4. Effect of different cations on acid and alkaline proteinase activity of *P.knowlesi*.

Cations (1mM)	% Activation /Inhibition	
	Acid proteinase	Alkaline proteinase
Ca^{2+}	-21.49	-16.50
Cu^{2+}	-25.23	-11.17
Cd^{2+}	-29.91	-2.91
Co^{2+}	+21.50	-3.40
Fe^{2+}	+3.74	-2.91
Fe^{3+}	-64.49	-38.84
Mg^{2+}	-85.98	-8.25
Mn^{2+}	-80.37	-12.14
Ni^{2+}	-82.24	-11.65
Zn^{2+}	-87.85	-55.34

The assay system contained 100 μ moles buffers of required pH, 20 μ moles cysteine hydrochloride, 0.05% (w/v) haemoglobin and suitably diluted enzyme, different cations in a final volume of 2.2 ml.

+ = Activation%, - = Inhibition%.

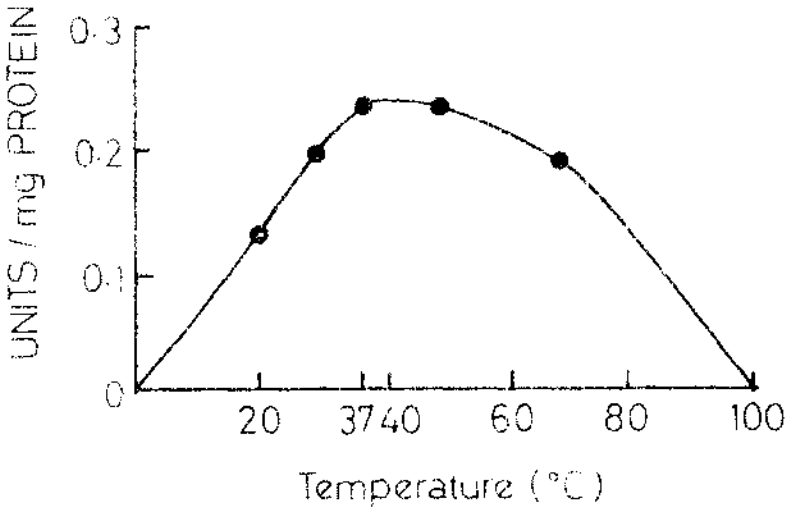


Fig. 2. Effect of different temperatures on acid proteases of parasite.

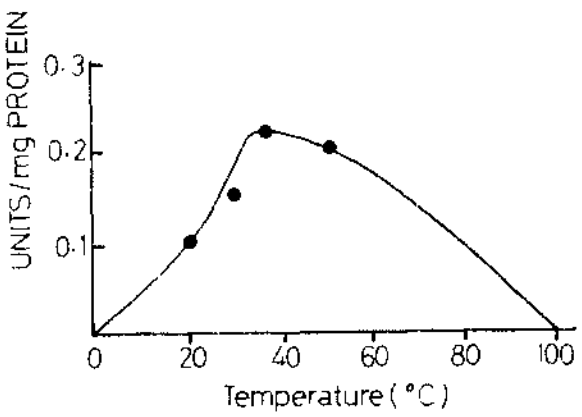
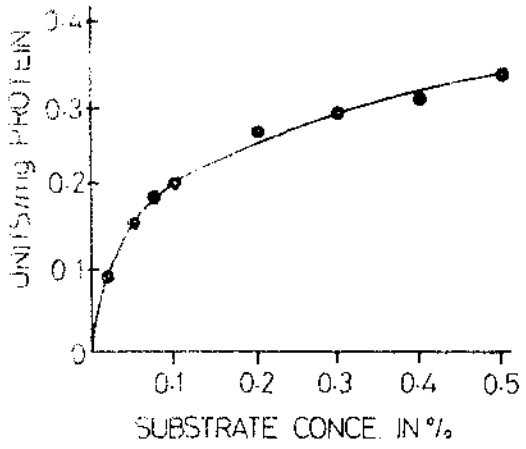
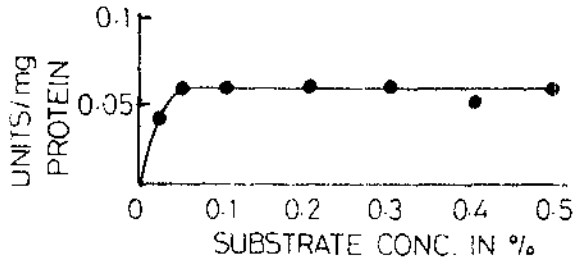
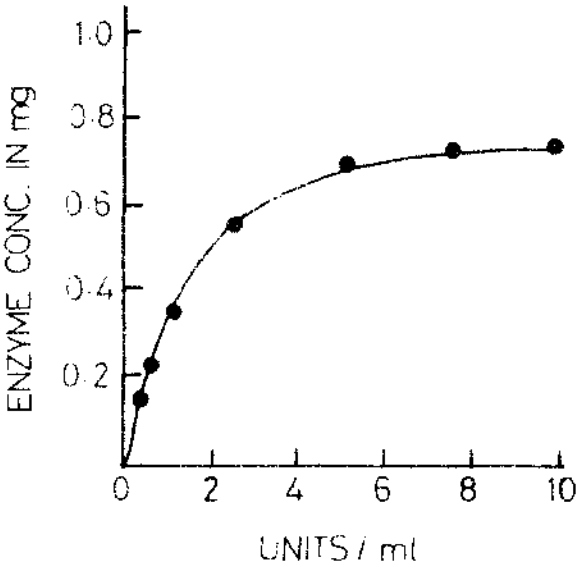


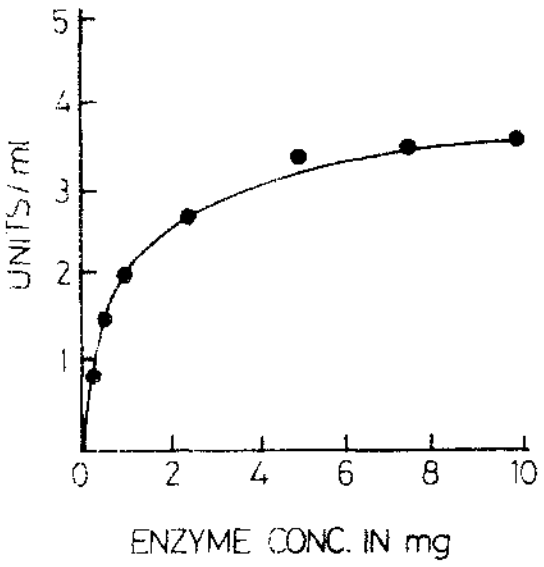
Fig. 3. Effect of different temperatures on alkaline proteases of parasite.





Figs. 4. and 5. Effect of substrate concentration on acid (Fig.4) and alkaline (Fig.5) proteases.





Figs. 6, and 7. Effect of different enzyme concentration. Acid proteinase (Fig.6) and alkaline proteinase (Fig.7).

moglobin concentration (Figs. 4 and 5). The rate of hydrolysis increased upto 4mg protein concentration of the enzyme and the increase was almost linear (Figs. 6 and 7).

The effect of cations on acid and alkaline proteinases was studied and data presented in Table 4 showed that divalent ions like Zn^{2+}, Mg^{2+}, Ni^{2+} and Mn^{2+} significantly inhibited 80% of the acid proteinase whereas Ca^{2+}, Cu^{2+} and Cd^{2+} produced 20-30% inhibition. Zn^{2+} inhibited 58% activity of alkaline proteinase while Fe^{3+} inhibited nearly 40% of alkaline and 65% of acid proteinase.

The effect of various activators and inhibitors on proteinases was also studied (Table 5). Metal chelating agents like EDTA, α-α' dipyridyl., 8-hydroxyquinoline and 0-phenanthroline had no inhibitory action on parasite proteinases, but sodium diethyldithiocarbamate had inhibitory action only on acid proteinase (64%). SH-group enzyme inhibitor iodoacetate had no effect on proteinases whereas 0-mercaptoethanol had slight

Table - 5. Effect of different activators or inhibitors on acid and alkaline proteinase activity of *P.knowlesi*

Additions (1 mM)	% Activation/Inhibition	
	Acid proteinase	Alkaline proteinase
EDTA	-14.29	+5.41
Diisopropylfluorophosphate	-12.70	+10.81
8-hydroxyquinoline	-4.76	-10.81
α,α'-Dipyridyl	-11.11	+13.51
O-mercaptoethanol	-19.05	-16.22
Iodoacetate	+0.00	-8.11
O-phenanthroline	-4.76	+21.62
Na-diethyldithiocarbamate	-63.49	+2.70
SB trypsin inhibitor (100 μg/ml)	+3.17	-89.19
SB trypsin inhibitor (200 μg/ml)	±0.00	-89.19
Without cysteine hydrochloride	-11.11	-16.22

Reaction mixture same as given under Table—4.
+ = Activation% ; - = Inhibition%.

Table 6. Effect of antimalarial drugs on acid and alkaline proteinase activity of *P.knowlesi*.

Drugs (1 mM)	% Activation/Inhibition	
	Acid proteinase	Alkaline proteinase
Chloroquine	-3.45	+9.84
Primaquine	+17.24	+3.28
Pyrimethamine	-1.72	-1.64
Amodiaquine	+3.45	+4.92
Mefloquine	-6.90	6.56
Quinine	1.72	-8.20
Mepacrine	±0.00	-1.64
Sulphadiazine	±0.00	+22.95
Sulphanilamide	±0.00	-13.11
Sulphaphenazole	-3.45	-9.84
Dapsone	+5.17	-8.20

The details of assay system are given under Table 4.
+ = Activation%; - = Inhibition%.

inhibitory effect (16%) on the alkaline and 19% on acid proteinases. Diisopropylfluorophosphate showed slight inhibitory effect on acid protease. Alkaline proteinase was markedly inhibited (89%) by soybean trypsin inhibitor while acid proteinase was not affected.

Different specific protease inhibitors were also tested for their action on parasite proteinases. Pepstatin at 20 μg/ml concentration inhibited nearly 80% of the acid proteinase activity while phosphoramidon at the same concentration inhibited 75% activity of alkaline proteinase. Chymostatin produced nearly 20% inhibition of both the proteinases at 20 μg/ml concentration. Elastinal at 20 μg/ml concentration inhibited only 27% activity of acid proteinase, whereas the alkaline proteinase was inhibited to an extent of 20% by 0.001 μg/ml concentration of this inhibitor.

The action of antimalarial drugs and antibiotics on the proteinases of the parasite was investigated and data presented in Tables 6 and 7, show that primaquine produced nearly 17% inhibition of acid proteinase while oxytetracycline had specific inhibitory action on alkaline proteinase (42%). Higher concentration (10^{-2}M) of the drugs and antibiotics could not be used as they interfered with the protein determination.

Table 7. Effect of antibiotics on acid and alkaline proteinase activity of *P.knowlesi*.

Antibiotics (1 mM)	% Activation/Inhibition	
	Acid proteinase	Alkaline proteinase
Tetrycline	+3.70	+3.85
Oxytetracycline	±0.00	-42.31
Doxycycline	-9.26	-5.77
Minocycline	-5.56	-5.77
Erythromycin	±0.00	-7.69
Kanamycin	+20.37	+3.85
Gentamycin	-9.26	-11.54
Ledermycin	-1.85	+88.46
Chloramphenicol	-7.41	+9.62

The details of the assay system are given under Table-4.
+ = Activation%; - = Inhibition%.

DISCUSSION

Cell-free parasite preparation of *P.knowlesi* has shown two pH optima at about pH 4.0 and 7.0. Cook *et al.* (1961) have also reported proteases maximally active at pH 5.0 and 8.0 in *P.knowlesi* and 4.0 and 8.0 in *P.berghei*. But Levy and his co-workers could demonstrate only acid proteinase with pH optima at 3.6 in *P.knowlesi* and *P.falciparum* (Levy *et al.*, 1974) and *P.berghei* (Levy and Chou, 1974). It may be so because the method of isolation of cell-free parasite was different in the two cases. About this Sherman (1977) wrote that "the fact Levy *et al.* did not recover an alkaline protease as did L.Cook is not surprising since the former workers used EDTA in their extracts and the alkaline protease was irreversibly inhibited by this Chelator."

Regarding the stability of proteinases, Cook *et al.* (1961) observed that most of the activity of enzyme was lost within 12 hrs at 2° C and Levy *et al.* (1974) found that the enzyme was stable. In the present study, it was observed that the enzyme at both the pH was fairly stable upto one month when stored at 20° C either in the form of intact parasites or parasite homogenate.

The results on the action of activators and inhibitors obtained in the present study are in agreement with Cook *et al.* (1961) who showed that alkaline proteinase was slightly inhibited by metal ions and metal chelates. These workers also reported that the enzyme was significantly inhibited by diisopropylfluorophosphate but that is not the case in the present study. Acid proteinase is inhibited by Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+} and Ni^{2+} and alkaline proteinase by Zn^{2+} and Fe^{3+} while cysteine hydrochloride stimulated both the enzymes. Specific inhibitors of proteases from the culture filtrates of actinomycetes, namely pepstatin (inhibitor of pepsin, gastrian, cathepsin D and rennin) (Umezawa *et al.*, 1970) chymostatin (inhibitor of cathepsin B and chymotrypsin but not trypsin) (Ikezawa *et al.* 1971; Umezawa *et al.*, 1970), leupeptin (inhibitor of trypsin but not chymotrypsin) (Aoyagi *et al.*, 1969), phosphoramidon (inhibitor of neutral metalloendopeptidases) (Suda *et al.*, 1973) and elastatinal (inhibitor of elastase (Umezawa *et al.*, 1973)) had some varying inhibitory action on both acid and alkaline proteinases. These results are in agreement with those of Levy *et al.* (1974). Soybean trypsin inhibitor specifically inhibited the alkaline proteinase of the parasite.

Acid and alkaline proteinase of *P.knowlesi* differ from the enzymes of erythrocyte membrane in some respects. At pH 7.4 stroma enzyme is not inhibited by Soybean trypsin inhibitor while the parasite enzyme is inhibited, and at pH. 7.4 and 3.2 the stroma enzyme is inhibited by cysteine hydrochloride while Zn^{2+} could not inhibit enzyme activity (Morrison and Neurath, 1953).

Chloroquine has been known to inhibit certain proteases (Cowley and Whitehouse, 1966; Woessner, 1969). However, different antimalarials (drugs and antibiotics) used in the present study did not inhibit proteases in *P.knowlesi* except primaquine and oxytetracycline which slightly inhibited acid and alkaline proteinase activity respectively.

From the above study it is concluded that the proteinases of *P.knowlesi* are a mixture of enzymes but the major part of acid proteinase appears to be cathepsin D like due to pH optima and its sensitivity towards pepstatin while alkaline proteinase may be trypsin or chymotrypsin-like as it is inhibited by Soybean trypsin inhibitor and chymostatin. The presence of cathepsin D as a greater part of acid proteinase has been reported previously in *P.knowlesi* and *P.falciparum* (Levy *et al.* 1974).

ACKNOWLEDGEMENT

The authors are grateful to Dr. Nitya Nand, Director, Central Drug Research Institute, for continued support and encouragement. Our thanks are due to the Council of Scientific and Industrial Research, New Delhi for the award of Junior Research Fellowship to one of the authors (HSB). The technical help rendered by Mr. K.L. Gulati is acknowledged.

REFERENCES

1. Aoyagi, T. Takeuchi, M. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda, and H. Umezawa, (1969). Leupeptins, New protease inhibitors from actinomycetes. *J.Antibiotics*, **22**: 283-286.
2. Anson, M.L. (1938). The estimation of pepsin, papain and cathepsin haemoglobin. *J.Gen. Physiol.*, **22**: 79-89.
3. Cook, J.., P.T. Grant, and W.O. Kermack, (1961). Proteolytic enzymes of the erythrocytic forms of rodent and simian species of malarial plasmodia. *Exp. Parasit.*, **11**: 372-379.
4. Cook, R.T., M. Aikawa, R.C. Rock, W. Little and H.Sprinz, (1969). The isolation and fractionation of *Plasmodium knowlesi*. *Milit.Med.*, **134** (sp.issue); 866-883.
5. Cowley, F.K. and M.W. Whitehouse, (1966). Biochemical properties of anti-inflammatory drugs. VII. Inhibition of proteolytic enzymes in connective tissue by chloroquine (Resochin) and related antimalarials/anti-rheumatic drugs. *Biochem. Pharmacol.*, **15**: 1071-1084.
6. Ikezawa H., T. Aoyagi, T. Takeuchi, and H. Umezawa, (1971). Effect of protease inhibitors of actinomycetes on lysosomal peptides hydrolases from swine liver. *J.Antibiotics.*, **24**: 488-490.
7. Kessel, J.F., W.P., Lewis, C.M. Pasquel, and J.A. Turner, (1965). Indirect hemagglutination and complement fixation test in amebiasis. *Am.J.trop.Med. Hyg.*, **17**: 540-550.
8. Levy, H.A. and S.C. Chou, (1973). Activity and some properties of an acid proteinase from normal and *Plasmodium berghei* infected red cells. *J. Parasitol.* **59**: 1064-1070.
9. Levy, M.R. and S.C. Chou, (1974). Some properties and susceptibility to inhibitors of partially purified acid proteases from *Plasmodium berghei* and from ghosts of mouse red cells. *Biochem.Biophys.Acta.*, **334**: 423-430.
10. Levy, M.R., W.A. Siddiqui, and S.C. Chou, (1974). Acid protease activity in *Plasmodium falciparum* and *P. knowlesi* and ghosts of their respective host red cells. *Nature*. (London), **247**: 546-549.

11. Lowry, O.H., N.J., Rosebrough, A.L. Farr, and R.J. Randall, (1951). Protein measurement with the folin phenol reagent. *J.Biol.chem.*, **193**: 265-275.
12. Morrision, W.L., and H. Neurath, (1953). Proteolytic enzyme of the formed elements of human blood.I. Erythrocytes. *J.Biol. Chem.*, **200**: 39-51.
13. Moulder, J.W. and E.A. Evans, (1946). The biochemistry of malaria parasite.VI. Studies on the nitrogen metabolism of the malaria parasite. *J.Biol. Chem.*, **164**: 145-157.
14. Sherman, I.W. (1977). Aminoacid metabolism and protein synthesis in malarial parasites. *Bull.WHO.*, **55**: 265-276.
15. Suda, H., T. Aoyagi, T. Takeuchi, and H. Umezawa, (1973). A thermolysin inhibitor produced by actinomycetes. Phosphoramidon. *J.Antibiotics*, **26**: 621-623.
16. Umezawa, H., T. Aoyagi, H. Morishima, H. Matsuzaki, M. Hamada, and T. Takeuchi, (1970). Pepstain, a new pepsin inhibitor produced by actinomycetes. *J.Antibiotics*, **26**: 259-262.
17. Umezawa, H., T. Aoyagi, A. Okura, H. Morishima, T. Takeuchi, and Y. Okame, (1973). Elastatinal, a new elastase inhibitor produced by actinomycetes. *J.Antibiotics*, **26**: 787-789.
18. Woessner, J.R., Jr. (1969). Lysosomal enzymes and connective tissue breakdown. In: *Inflammation Biochemistry and Drug Interaction*. Ed. Berteli, A. and Houck, J.C., *Excerpta Med. Found. Amsterdam*, pp. 122-130.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 83-90

Genetics of a Sex Linked and Two Autosomal Mutants in Species B of the Taxon *Anopheles culicifacies* Giles

SARALA K. SUBBARAO, I. ADAK, K. VASANTHA and V.P. SHARMA

Three mutants, *white-eye (w)*, *Red-thorax (Rt)* and *creamish-larva (cr)*, were isolated from laboratory strains of *Anopheles culicifacies* species B. Genetic crosses of *w* strain established that *w* is a sex linked recessive gene which expresses in homozygous condition in females and in hemizygous condition in males. *Red-thorax* was found to be an autosomal dominant mutant and *creamish-larva* an autosomal recessive mutant. Linkage studies between the two autosomal mutants placed the two mutants in separate linkage groups. Thus in species B, three linkage groups have been identified corresponding to the three cytologically observed chromosomes.

INTRODUCTION

A. culicifacies is a major malaria vector transmitting 60-70% of malaria in the Indian sub-continent. It is a widely distributed species and has been incriminated from most of the areas of its distribution.

Successful colonization of *A. culicifacies* (Ainsley, 1976; Ansari *et al.*, 1977) has led to intensive research on genetical aspects. Such studies are of fundamental importance in understanding the biology of the species.

Polytene chromosome studies revealed the existence of two sibling species; species A and species B in this taxon (Green and Miles, 1980). Now, another method has been found for the identification of the sibling species through the morphological differences in male metaphase chromosomes i.e., the Y-chromosome in species A is submetacentric while in species B, it is acrocentric (Vasantha *et al.*, 1982).

The importance of the isolation of phenotypic markers and their genetic studies has been unequivocally demonstrated in all vector species (Kitzmiller and Laven, 1958; French *et al.*, 1963; Todano, 1970; Aslamkhan, 1973; Curtis, 1977; Sakai *et al.*, 1977 and 1979; Munstermann and Craig, 1979; Sharma *et al.*, 1979). To date, only four phenotypic markers have been isolated and studied in *A. culicifacies* (Sakai *et al.*, 1977, 1979, and 1981; Sakai and Baker, 1980). The Sattoki strain from which these mutants were isolated was identified as species A (Green and Miles, 1980). This paper presents the genetics of two autosomal mutants and one sex linked mutant from species B.

Accepted for publication, 9 November 1982.

Malaria Research Centre (ICMR),
22 Shani Nath Marg,
Delhi-110054.

MATERIAL AND METHODS

The following stocks were used in the study.

White-eye (w): White-eyed male pupae were observed in the rearing pans of Sirolifarm (U.P. Terai) laboratory strain. The pupae were isolated and crossed with wild type females. The progeny were inbred and white eyed male and female pupae were isolated in the F_2 generation and a true-breeding white eyed colony was established. The mutant expresses its phenotype, white eyes, from the I instar through pupae to adult stages. This mutant, in addition to its phenotypic expression in eyes, also expresses in the form of reduced body pigmentation in the larvae and pupae. Thus, these mutants can easily be distinguished from wild type as early as in the II instar larval stage. The phenotypic expression of this mutant appears to be similar to that of *colorless-eye* of *Anopheles stephensi* (Sharma *et al.* 1977 and Subbarao & Adak, 1978).

Red-thorax (Rt): Red thorax larvae were first observed in Gopalpur (Gujarat) and later in Aurangabad (Maharashtra), Okhla (Delhi) and Sirolifarm (U.P. Terai) laboratory strains. Red thorax larvae were isolated and a pure colony was established after 2-3 generations of inbreeding and selection.

The mutant expresses its phenotype at the late III larval instar as a red patch on the dorsal side of the entire thorax. The colouration becomes more prominent as the larvae go through the IV instar and the pupal stages. The mutant adults are, however, not distinguishable from the wild type mosquitoes.

Creamish-larva (cr): Creamish coloured larvae were observed in Okhla (Delhi) strain. Creamish larvae were isolated and a colony which bred true to the phenotype was established.

The mutant expresses its phenotype in all larval instars. However, the expression becomes prominent from the II instar onwards and can easily be distinguished from the wild type at this stage. The pupae and adult mosquitoes are not distinguishable from the wild ones. The laboratory strains from which the above mentioned mutants were isolated were all cytologically identified as species B (Subbarao *et al.*, 1980). Crossing experiments were carried out in 30×30×30 cm cloth cages in a laboratory maintained at 27-28°C and 70-80% R.H. Rearing of the mosquitoes was carried out following Ansari *et al.*, 1977 and 1979.

RESULTS & DISCUSSION

Inheritance pattern of *white-eye*: Results from genetic crosses between *white-eye* (*w*/*w*) and wild type (+/+) are summarized in Table 1. In the progeny of cross 1, (white-eye females × wild type males), females were of wild type and males were of mutant phenotype, while in the reciprocal cross (cross 2) both females and males were of wild type. These results suggested that the gene for white-eye phenotype is recessive and may be sex-linked.

F_1 progeny of the reciprocal crosses were inbred to obtain F_2 (crosses 3 and 4). In cross 3 (inbreeding of progeny from cross 1), wild type and white eyed phenotypes were observed in both the sexes. The four categories were observed in a ratio of 1:1:1:1 ($P<0.05$). In cross 4 (F_1 inbreeding of cross 2), white eyed females were absent while the wild type females, wild type males and white eyed males were in a ratio of 2:1:1 ($\chi^2 = 2.38$)

When F_1 females from either reciprocal crosses (cross 1 or 2) were crossed to wild type males, the resulting progeny were of three categories: wild type females, wild type males and white males (crosses 5 & 6). In cross 5, the three categories were not close to the expected ratio of 2:1:1 ($P<0.001$) but in cross 6, the observed numbers were in an expected ratio of 2:1:1 ($\chi^2 = 3.13$). When F_1 males from either reciprocal cross (crosses 1 & 2) were crossed to wild type males, the resulting progeny were all wild type (crosses 7 & 8).

Backcrossing of F_1 females (from crosses 1 & 2) to white-eye males resulted in four categories of progeny: wild type females, white females, wild type males and white males (cross 9 & 10). Even though in cross 9 all the four categories were present, the ratio was not in a perfect 1:1:1:1 ratio ($P<0.001$) due to significantly smaller numbers of white eyed progeny compared to wild type. However, in cross 10 the four categories were in a 1:1:1:1 ratio ($\chi^2 = 2.17$).

The back cross of F_1 males from cross 1 to *white-eye* females (cross 11) produced all white eyed progeny.

Presence of four categories of progeny in crosses 3, 9 & 10 and absence of white eyed females in crosses 4, 5 and 6 strongly supports the idea that the gene for *white-eye* is sex linked and recessive in females and hemizygous in males. Thus, these results together with the results from crosses 7, 8 and 11 clearly indicated that the gene for *white-eye* is located on the X-chromosome and females are XX type and males XY type with no *white-eye* gene on Y-chromosome. Failure to obtain different categories of progeny close to expected 2:1:1 ratio in cross 5 and 1:1:1:1 ratio in cross 9 appears to be due to poor survival of individuals with white-eyes in these crosses. In these two crosses the sexes were not in a perfect 1:1 ratio as in the other crosses.

Table 1 Results elucidating the inheritance pattern of *white-eye (w)* in *A. cultifacies* species B

	Presumptive Parental genotypes			Progeny phenotype				Chi-square testing for	
				Wild		White		1:1 sex ratio	phenotypes
	♀		♂	♀	♂	♀	♂		
1.	*w/w*	×	+/-	451 (447)	0	0	443 (447)	0.06 n.s.	
2	+/+	×	w/-	1703 (1687)	1671 (1687)	0	0	0.30 n.s	
3.	*w/+*	×	w/-	444 (400.5)	412 (400.5)	408 (400.5)	365 (400.5)	1.43 n.s	8.34*
4	+/*w*	×	+/-	457 (458)	213 (229)	0	246 (229)	0.001 n.s	2.38 n.s
5.	*w*/+	×	+/-	686 (606)	367 (303)	0	159 (303)	20.1***	92.4***
6	+/*w*	×	+/-	206 (203)	112 (101.5)	0	88 (101.5)	0.08 n.s	3.12 n.s
7	+/+	×	w/-	108 (101)	94 (101)	0	0	0.97 n.s	
8	+/+	×	+/-	257 (247)	237 (247)	0	0	0.81 n.s	
9	*w*/+	×	w/-	158 (120.8)	112 (120.8)	106 (120.8)	107 (120.8)	4.18*	14.52***
10	+/*w*	×	w/-	76 (67.5)	70 (67.5)	64 (67.5)	60 (67.5)	1.78 n.s	2.17 n.s
11	*w*/*w*	×	w/-	0	0	74 (68.5)	63 (68.5)	0.88 n.s	

* $P < .05$

*** $P < .001$

n.s. = Not significant

( ) = Expected Nos.

Inheritance pattern of Red-thorax (Rt): Results from genetic crosses between *Red thorax* and wild type strains are presented in Table 2. In reciprocal crosses (crosses 1 and 2), F_1 progeny were of mutant phenotype and the two sexes were in a ratio of 1:1. The fact that both the sexes of F_1 progeny (from crosses 1 and 2) were of the mutant phenotype strongly suggests that it could be a dominant mutant. Inbreeding of F_1 progeny of the two parental crosses resulted in two phenotypic categories: larvae with red thorax and wild type larvae (crosses 3 and 4). In cross 3, the two sexes and the two phenotypic categories were not in a perfect expected ratio of 1:1 and 1:3 respectively but in cross 4, the sexes were in 1:1 ratio (χ^2=2.6) and the two phenotypes were in the expected 3:1 ratio (χ^2=1.29). Backcrossing of the F_1 progeny to the red thorax stock produced all progeny with red thorax (crosses 5, 6, 7 and 8) and backcrossing to the wild type stock produced two phenotypes: red thorax and wild in 1:1 ratio (crosses 9, 10, 11 and 12). In three crosses, 10, 11 and 12, the sex and phenotypic categories were in the expected 1:1 ratio while in cross 9 the phenotypic categories were not in a perfect 1:1 ratio ($P<0.01$).

The results from inbreeding and backcrosses of F_1 progeny confirm that *Red thorax* is an autosomally inherited dominant mutant. This is the first dominant phenotypic marker reported in *A. culicifacies*.

Inheritance Pattern of creamish-larva (cr): Results from genetic crosses between creamish and wild type strains are presented in Table 3. F_1 progeny of crosses 1 and 2 were all of wild type and the sex ratio was approximately 1:1. Inbreeding of F_1 progeny from the two parental crosses produced wild type and mutant progeny in a ratio of 3:1 (crosses 3 and 4) with chi square values of 1.42 and 0.55 respectively. Backcrossing of the F_1 progeny with creamish strain produced wild and creamish phenotypes in a 1:1 ratio (crosses 5, 6, 7 and 8) while the backcrosses with wild type strain produced only wild type progeny (crosses 9, 10, 11 and 12).

The fact that F_1 progeny in both the crosses (1 and 2) were wild type suggested that the *creamish-larva* could be an autosomally inherited recessive mutant. Results from inbreeding and backcrossing of F_1 progeny confirmed this.

Linkage: To establish the linkage relationship between the two autosomally inherited mutants, *Red thorax* and *creamish-larva*, reciprocal crosses were made. F_1 progeny of the reciprocal crosses expressed red thorax phenotype confirming the earlier results that *Red-thorax* was a dominant mutant. Inbreeding of the F_1 progeny (crosses 3 & 4) resulted in four phenotypes: (i) red thorax (ii) wild (iii) creamish and (iv) creamish larvae with red thorax in a ratio of 9:3:3:1 respectively. Backcrossing of F_1 progeny with creamish strain yielded three phenotypes: red thorax, wild and creamish in a ratio of 1:1:2 respectively (crosses 5, 6 & 7).

The double mutant phenotype, creamish larva with red thorax, obtained in crosses 3 and 4 were inbred to establish a colony. This phenotype bred true. Females from this stock when crossed with wild type males produced progeny with red thorax (cross 8). When F_1 progeny of this cross (cross 8) were backcrossed to *Red thorax* strain, it produced red thorax progeny (cross 9). Backcrossing of F_1 progeny with double mutant strain produced three phenotypic categories: larvae with red thorax, creamish larvae and creamish larvae with red thorax in a ratio of 2:1:1 respectively (cross 10).

Presence of four phenotypic catagories among the F_2 progeny (crosses 3 and 4) strongly suggested that the two mutants, *Rt* and *cr*, are in different linkage groups. This was also confirmed by back crosses (5, 6, 7, 9 and 10).

Red thorax expressed itself in heterozygous condition, when crossed with wild type and creamish strains. However, it failed to express in the heterozygous condition in the creamish background i.e., when the progeny genotype was Rt +; *cr*/*cr*. This was evident from the inbreeding of creamish larvae of crosses 3 and 4 which resulted in segregation of two phenotypes: 1) Creamish and 2) Creamish larvae with red thorax. The fourth phenotypic category, creamish with red thorax, of crosses 3 and 4 bred true when inbred. This shows that when the

Table 2 Results of crosses between *Red-thorax* (*Rt*) and wild type in *M. cultriformis* species B.

Presumptive Parental genotypes ♀		♂	Progeny phenotypes: Red thorax ♀	Red thorax ♂	Red thorax Total	Wild ♀	Wild ♂	Wild Total	Chi-square testing for 1:1 ratio ♀:♂	Chi-square testing for 1:1 ratio Rt:+	Chi-square testing for 3:1 ratio Rt:+
1. +/+	×	*Rt*/*Rt*	804	771	1575	0	0	0	0.7 n.s		
2. *Rt*/*Rt*	×	+/+	829	806	1635	0	0	0	0.3 n.s		
3. +/*Rt*	×	+/*Rt*	329	241	570	152	116	268	18.3***		21.7***
4. *Rt*/+	×	*Rt*/+	199	179	378	79	62	141	2.6 n.s		1.29 n.s.
5. +/*Rt*	×	*Rt*/*Rt*	396	337	733	0	0	0	4.7*		
6. *Rt*/*Rt*	×	+/*Rt*	203	165	368	0	0	0	3.92*		
7. *Rt*/+	×	*Rt*/*Rt*	327	378	605	0	0	0	3.96*		
8. *Rt*/*Rt*	×	*Rt*/+	337	326	663	0	0	0	0.18 n.s		
9. +/*Rt*	×	+/+	154	162	316	208	180	388	0.56 n.s	7.4**	
10. +/+	×	+/*Rt*	194	197	391	199	169	368	0.96 n.s	0.7 n.s	
11. *Rt*/+	×	+/+	520	499	1019	544	524	1068	0.84 n.s	2.40 n.s	
12. +/+	×	*Rt*/+	269	275	544	294	310	604	0.42 n.s	3.2 n.s	

* = P <0.05
** = P <0.01
*** = P <0.001
n.s. = not significant.

Table 3 Results of crosses between *cr* (*cream-larva locus*) and wild type in *A. culicifacies* species B

Cross No.	Presumptive Parental genotypes ♀	×	♂	Progeny phenotypes: creamish ♀	creamish ♂	creamish Total	wild ♀	wild ♂	wild Total	Chi-square testing for 1:1 ratio ♀ : ♂	1:1 ratio + : *cr*	3:1 ratio + : *cr*
1.	+/+	×	*cr*/*cr*	0	0	0	664	671	1335	0.04 n.s.		
2.	*cr*/*cr*	×	+/+	0	0	0	609	519	1128	7.2**		
3.	+/*cr*	×	+/*cr*	94	84	178	270	288	558	0.08 n.s.		1.42 n.s.
4.	*cr*/+	×	*cr*/+	78	70	148	226	250	476	0.40 n.s.		0.55 n.s.
5.	+/*cr*	×	*cr*/*cr*	214	212	426	242	231	473	0.18 n.s.	2.46 n.s.	
6.	*cr*/*cr*	×	+/*cr*	128	128	256	137	148	285	0.02 n.s.	1.4 n.s.	
7.	*cr*/+	×	*cr*/*cr*	114	121	235	157	144	301	0.06 n.s.	8.12**	
8.	*cr*/*cr*	×	*cr*/+	316	307	623	352	330	682	0.72 n.s.	2.6 n.s.	
9.	+/*cr*	×	+/+	0	0	0	490	459	949	1.06 n.s.		
10.	+/+	×	+/*cr*	0	0	0	242	236	478	0.07 n.s.		
11.	*cr*/+	×	+/+	0	0	0	416	393	809	0.65 n.s.		
12.	+/+	×	*cr*/+	0	0	0	312	305	617	0.08 n.s.		

** = $P < 0.01$

n.s. = not significant.

Table 4 Linkage relationship between *Red-thorax (Rt)* and *creamish-larva (cr)* in *A. culicifacies* species B.

Cross No.	Presumptive Parental genotypes ♀		♂	Progeny Phenotypes: red thorax	wild	crea-mish	creamish larvae with red patch	Total	Chi-square value
1.	*Rt/Rt; +/+*	×	*+/+; cr/cr*	594	0	0	0	594	–
2.	*+/+; cr/cr*	×	*Rt/Rt; +/+*	1191	0	0	0	1191	–
3.	*Rt/+; +/cr*	×	*Rt/+; +/cr*	380 (389.7)	162 (129.9)	113 (129.9)	38 (43.3)	693	11.01*
4.	*+/Rt; cr/+*	×	*+/Rt; cr/+*	235 (224.1)	69 (74.8)	70 (74.8)	25 (24.9)	399	1.29 n.s.
5.	*Rt/+; +/cr*	×	*+/+; cr/cr*	132 (129.5)	119 (129.5)	267 (259)	0	518	1.15 n.s.
6.	*+/+; cr/cr*	×	*Rt/+; +/cr*	231 (222.8)	191 (222.8)	469 (445.5)	0	891	6.83 n.s.
7.	*+/Rt; cr/+*	×	*+/+; cr/cr*	91 (97.3)	104 (97.3)	144 (144.5)	0	389	0.87 n.s.
8.	*Rt/Rt; cr/cr*	×	*+/+; +/+*	738					
9.	*Rt/+; cr/+*	×	*Rt/Rt; +/+*	1363					
10.	*Rt/Rt; cr/cr*	×	*Rt/+; cr/+*	462 (436.5)		239 (218.25)	172 (218.25)	873	13.1*

( ) = Expected nos.

* = $P < .05$

n.s. = Not significant.

larvae are homozygous for *cr*/*cr*, not just a single dose of *Rt*, but two doses of *Rt* are required for its expression.

All the three mutants have excellent penetration. Because of the sexlinked inheritance and excellent penetration, the *white eye* could be used in studying the genetic nature of the mechanism of hybrid sterility in the complex. Thus, this study establishes three linkage groups corraborating the cytologically observed three pairs of chromosomes (Vasantha *et al.*, 1982) in species B.

ACKNOWLEDGEMENT

We gratefully acknowledge the technical assistance of Mr. K.B. Masiwal & Mr. H.D. Joshi and Mr. Naresh Kumar Sharma for typing the manuscript.

REFERENCES

1. Ainsley, R.W. (1976). Laboratory colonization of the malaria vector, *Anopheles culicifacies*. *Mosq. News.*, **36**: 256-258.
2. Ansari, M.A., T.R. Mani and V.P. Sharma (1977). A preliminary note on the colonization of *Anopheles culicifacies* Giles. *J.Com.Dis.* **9**: 206-207.
3. Ansari, M.A., V.P. Sharma and R.K. Razdan (1979). Mass rearing procedure for *Anopheles stephensi* Liston. *J.Com. Dis.* **10(2)**: 131-135.
4. Aslamkhan, M. (1973). Sex chromosome and sex determination in the malaria mosquito *Anopheles stephensi*. *Pak.J. Zool.* **5**: 127-130.
5. Curtis, C.F. (1977). Hybrid sterility in the *Anopheles gambiae* complex: mechanism and possible means of using it for genetic purposes. Medical Entomology Centennary Symposium Proceedings, Royal Society Tropical Medicine and Hygiene.
6. French, W.L. and J.B. Kitzmiller (1963). Tests for multiple fertilization in *Anopheles quadrunaculatus*. Proc. Fifth annual meeting of the New Jersey Mosquito extermination association and the nineteenth annual meeting of the American Mosquito Control Association : 374-380.
7. Green, C.A. and S.J. Miles (1980). Chromosomal evidence for sibling species of the malaria vector *Anopheles* (cellia) *culicifacies* Giles *J.Trop.Med.Hyg.* **83**: 75-78.
8. Kitzmiller, J.B. and H. Laven (1958). Tests for multiple fertilization in Culex mosquitoes by the use of genetic markers. *Amer.J. Hyg.* **67**: 207-213.
9. Munstermann, L.E. and G.B. Craig, Jr. (1979). Genetics of *Aedes aegypti*. *J. Heredity* **70**: 291-296.
10. Sakai, R.K., R.W. Ainsley and R.H. Baker (1977). The inheritance of *Rose-eye*, a sex linked mutant in the malaria vector *Anopheles culicifacies*. *Can.J.Genet.Cytol.* **19**: 633-636.
11. Sakai, R.K., and M. Ahmad, F. Aslam and R.H. Baker (1979). Genetics of two new autosomal loci in linkage group II of the malaria vector *Anopheles culicifacies*. *Ann. Entomol. Soc. Am.*, **72(3)**: 328-330.
12. Sakai, R.K. and R.H. Baker (1980). The Genetics of *white-eye* in *Anopheles culicifacies*. *Mosq. News* **40**: 23-26.
13. Sakai, R.K., R.H. Baker and C.J. Dubash (1981). The inheritance of *golden body*, a sex linked mutant in the malaria vector *Anopheles culicifacies*. *Can. J. Genet. Cytol.* **23**: 579-583.
14. Sharma, V.P., T.R. Mani, T. Adak and M.A. Ansari (1977). *Colourless-eye* a recessive autosomal mutant of *Anopheles stephensi*. *Mosq. News.* **37(4)**: 667-669.
15. Sharma, V.P., Sarala K. Subbarao, M.A. Ansari and R.K. Razdan (1979). Inheritance pattern of two new mutants *red-eye* and *greenish brown larva* in *Anopheles stephensi*. *Mosq. News* **39(3)**: 655-657.
16. Subbarao, Sarala K. and T. Adak (1978). Genetic analysis of a larval color mutant green larva in *Anopheles stephensi*. *Mosq. News.* **38(1)**: 51-53.
17. Subbarao, Sarala K., T. Adak and V.P. Sharma (1980). *Anopheles culicifacies*: sibling species distribution and vector incrimination studies. *J.Com.Dis.*, **12(2)**: 102-104.
18. Tadano Takeo (1970). Recombination values between the *white-eye* and factors for resistance to Dieldrin, Abate, Fenthion and Fenitrothion in larva of *Culex pipiens* pallens Coquillett. *Japanese J. Sanitary Zoology* **21(3)**: 156-160.
19. Vasantha, K., Sarala K. Subbarao, T. Adak and V.P. Sharma (1982). Karyotypic variations in *Anopheles culicifacies* sibling species complex. *Ind.J.Malariol* **19(1)**, 27-32.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 91-96

Induction of Pyrimethamine Resistance in a Mefloquine Resistant Strain of *Plasmodium berghei*

S.K. PURI and G.P. DUTTA

A mefloquine resistant strain of *Plasmodium berghei* was subjected to interrupted subcurative doses of pyrimethamine followed by increasing drug pressure for 18 passages in weanling rats which resulted in the selection of a strain resistant to both mefloquine and pyrimethamine. The level of resistance of this strain to mefloquine was 256 mg/kg and pyrimethamine 128 mg/kg, administered from day 0 to +4. Besides, the strain showed high degree of cross-resistance to quinine and metakelfin, and moderate level of resistance to 4 aminoquinolines, mepacrine and sulfanilamide. However, the strain maintained its sensitivity to dapsone, primaquine and sulfadiazine. Drug free passages of the resistant strain for 70 days resulted in the loss of mefloquine resistance while the pyrimethamine resistance was maintained. Along with the loss of mefloquine resistance on drug free passages, the cross resistance of this strain to quinine and 4-aminoquinolines was also lost.

INTRODUCTION

The spread of chloroquine-resistant *P. falciparum* in India and other south-east Asian countries is posing a serious problem and in certain areas over 90% infections are reported to be resistant to chloroquine (WHO, 1981). Today mefloquine is the only single drug effective against chloroquine and multiple drug resistant parasites, there is need to assess whether mefloquine strains of *P. falciparum*. In view of the unique activity of mefloquine against resistant parasites, there is need to assess whether mefloquine resistant strain can acquire resistance to other antimalarials or not. Since sensitive strain of *P. berghei* was found earlier (Agarwal *et al.* 1979) to readily develop pyrimethamine resistance, attempt has been made in this study to superimpose pyrimethamine resistance on a mefloquine resistant strain.

MATERIAL AND METHODS

The mefloquine resistant strain of *P. berghei* (Kazim *et al.* 1979) selected by serial blood passage in weanling rats (Druckery strain; 18-22 gm.) was cryopreserved between 1979-1981 and since then maintained under constant drug pressure. In the present study, this mefloquine resistant strain was exposed to increasing drug pressure of pyrimethamine in successive passages, to select a strain resistant to both mefloquine and pyrimethamine. Giemsa stained blood smears were used for recording the number of parasitized cells/10,000 RBC. The level of resistance, the stability of resistance after drug-free passage and the cross-sensitivity to other antimalarials was determined on MED basis (Agarwal *et al.*,1979). All the drugs were administered orally. Mefloquine hydrochloride was supplied by M/s. Roche Products Ltd., Basel. The other antimalarial drugs used viz: pyrimethamine, chloroquine, amodiaquine, mepacrine, quinine, primaquine, sulfadiazine, sulfanilamide, dapsone and metakelfin (a combination of 25 mg pyrimethamine plus 500 mg sulfamethopyrazine) were obtained commercially.

Accepted for publication, 9 November 1982.
Division of Microbiology,
Central Drug Research Institute,
Lucknow.

RESULTS

Selection of resistant strain: The study was initiated with the strain of *P. berghei* resistant to mefloquine at a daily dose of 256 mg/kg administered from day 0 to +3. Table 1 presents the sequential steps in the induction of pyrimethamine resistance by subcurative therapy. Initiating the pyrimethamine treatment with 0.5 mg/kg dose, which is half of the daily MED, the drug pressure was gradually increased during the subsequent passages depending on the appearance of parasites in treated animals. By 9th passage, the parasites were partially resistant to 128 mg/kg dose of pyrimethamine

Table 1 – Induction of pyrimethamine resistance in a mefloquine resistant strain of *Plasmodium berghei*.

Sl. passage No.*	Duration of passage (Days)	Drugs administered				Total pyrimethamine admn. (mg/kg)	Parasites /10^6 RBC (on days)						
		Pyrimethamine mg/kg	on days	Mefloquine mg/kg	on days		5	6	7	8	9	10	11
		Nil		256	1-4		320	780	1100	1530	2070		
1	0-10	0.5	4,6,8	--	--	1.50	190	570	330	315	225	430	
2	11-23	1.0	3,5,7	--		4.00	250	180	460	120	150	120	70
3	24-30	1.0	4-7	--	--	4.00	570	580	410				
4	31-43	2.0	2,6-10	--		12.00	40	115	70	70	190	80	50
		2.0	3,4,6	128	4,6,	10.00	110	90	120	150	360	520	750
			11,12		11								
5	44-52	4.0	6-8			12.00	190	260	370	280	610		
		4.0	5-8	128	6-8	16.00	110	330	540	410	180		
6	53-61	8.0	3-8			48.00	230	360	140	145	140		
		16.0	4-8	--	--	80.00	430	350	310	160	320		
		32.0	5-8			128.00	520	410	230	110	100		
7	62-71	64.0	2,3,6,7		-	256.00	50	140	70	80	170	330	
		64.0	4-7	128	4-7	256.00	330	400	128	145	90	217	
8	72-83	64.0	0-3			256.00	-ve	-ve	-ve	-ve	-ve	-ve	
		64.0	6-9	128	6-9	256.00	195	480	280	260	450	80	50
9	84-94	128.0	6-9	128	6-9	512.00	240	170	70	50	20	20	
		128.0	6-9	256	6-9	512.00	380	150	110	40	10	20	
10	95-101	128.0	0-3	256	0-3	512.00	-ve	-ve	-ve	-ve	-ve	-ve	
		128.0	1-4	256	1-4	512.00	80	270					
11	102-106						380						
12	107-114	96.0	4-6	256	4-7	288.00	410	220	115	35			
		128.0	3-6	128	3-6	512.00	520	460	230	270			
18	161-168	128.0	0-4	256	0-4	640.00	270	580	350				
		128.0	0-4	--	--	640.00	170	320	220				
		---		256	0-4		230	360	220				

*The mefloquine resistant parasites were maintained in weanling rats and exposed to interrupted subcurative doses of pyrimethamine alone or in combination with mefloquine.

administered from day +6 to +9. The resistance at this level was incomplete as this dose eliminated all parasites when treatment was given from day 0 to +3. In 18th passage, the parasites were fully resistant to 128 mg/kg dose of pyrimethamine administered from day 0 to +4, the level of resistance thus being 128-folds.

Since mefloquine resistance is known to be unstable factor, the drug pressure was maintained at intervals of 2-3 passages. The resultant parasites obtained after the 18th passage were resistant to 128 mg/kg pyrimethamine and 256 mg/kg mefloquine administered concurrently from day 0 to +4. The strain has since been maintained upto 45th passage in presence of both the drugs.

Stability of resistance: A subline initiated from the 25th passage of mefloquine-pyrimethamine resistant strain was maintained without drug pressure for 9 passages in rats over a period of 70 days. The parasites were then subjected to treatment with either of the two drugs to check the stability of resistance. Although the parasites retained resistance upto 128 mg/kg dose for pyrimethamine, the resistance to mefloquine was lost and the tests showed that the parasite had become sensitive to 8 mg/kg/day dose of mefloquine.

Sensitivity/cross resistance to other drugs: The chemotherapeutic response of the following four strains have been compared in Table 2.

A. Sensitive (normal) strain of *P.berghei*
B. Mefloquine resistant strain derived from the original mefloquine resistant strain described by Kazim *et al.* (1979), which was further exposed to mefloquine for over a year. Its sensitivity to some of the drugs has been altered since our earlier report (Kazim *et al.*,1979);
C. Mefloquine-pyrimethamine resistant strain reported in this study;
D. Pyrimethamine resistant strain derived from C above after drug-free passages.

The mefloquine and mefloquine-pyrimethamine resistant parasites have been found to exhibit high level of cross-resistance to quinine (upto maximum tolerated dose), 8-fold resistance to chloroquine and amodiaquine and 2-fold resistance to mepacrine. The mefloquine-pyrimethamine resistant strain in addition also shows 4-fold resistance to sulfanilamide.

The antimalarial activity of metakelfin against the various strains has also been compared. This drug combination has shown strong synergistic action against both the sensitive as well as the mefloquine resistant strain. Both these strains were completely suppressed at a dose of 0.01 mg/kg pyrimethamine plus 0.20 mg/kg sulfamethopyrazine. On the other hand, the mefloquine-pyrimethamine resistant strain has developed 17-fold resistance to metakelfin.

DISCUSSION

The present study has shown that mefloquine resistant strain of *P.berghei* can readily acquire a high level of pyrimethamine resistance. The combined mefloquine-pyrimethamine resistant strain developed in this study also shows a high level of cross-resistance to quinine and moderate resistance to 4-aminoquinolines, mepacrine and sulfanilamide. Unlike the mefloquine resistant strain, this mefloquine-pyrimethamine resistant strain has developed 17-fold resistance to metakelfin, though the strain continues to be sensitive to dapsone, sulfadiazine and primaquine.

Peters *et al.* (1977) have proposed that when mefloquine was combined with pyrimethamine or sulfaphenazole or primaquine, it markedly slowed down the development of resistance to mefloquine in *P.berghei*. Similarly Merkli *et al.* (1980) suggested that the use of a mixture of mefloquine and Fansidar considerably reduced the possibility of development of resistance in *P.berghei* to either of these drugs. We reported earlier that pyrimethamine resistant strain of *P.berghei* could be readily selected if the parasite was exposed to subcurative doses of pyrimethamine (Agarwal *et al.*, 1979). The evidence presented in this study shows that pyrimethamine resistance could be easily superimposed on the strain already resistant to mefloquine and the resultant strain acquires compound resistance to maximum tolerated doses of either of these drugs.

Table 2 Sensitivity (MED. daily dose mg/kg) of normal (sensitive) and drug resistant strains of *P. berghei* to various antimalarial drugs.

Drug	Sensitive strain A	Mefloquine resistant strain B	Mefloquine pyrimethamine resistant strain C	Pyrimethamine resistant strain D
Chloroquine	8.0	64.0	64.0	8.0
Amodiaquine	8.0	64.0	64.0	8.0
Quinine	300.0	>900.0	>900.0	300.0
Mepacrine	10.0	20.0	20.0	10.0
Mefloquine	4.0	>256.0	>256.0	4.0
Pyrimethamine	1.0	1.0	>128.0	>128.0
Primaquine	17.5	17.5	17.5	17.5
Sulfadiazine	0.4	0.4	0.4	0.4
Dapsone	4.0	4.0	4.0	4.0
Sulfanilamide	100.0	100.0	400.0	400.0
(Pyrimethamine	0.01	0.01	0.17	0.08
Metakelfin (				
(+	+	+	+	+
(				
(Sulfamethopyrazine	0.20	0.20	3.40	1.60

A = Parent sensitive strain of *P. berghei*

B = Strain derived from the mefloquine resistant strain reported by Kazim *et al.* (1979) after further exposure to mefloquine.

C = Strain selected from B during the present study.

D = Strain derived from C after drug free passages.

We have not exposed the sensitive strain to subcurative low dose combination of mefloquine and pyrimethamine to see whether pyrimethamine slows down the emergence of mefloquine resistance as was done by Peters *et al.* (1977). However, our study clearly shows that if mefloquine resistance emerges in the field, the use of pyrimethamine alone for the control of mefloquine resistant parasite would not be safe because there would be possible danger of building up of compound resistance. Similarly cross-resistance to quinine observed in our mefloquine resistant strain as well as in mefloquine-pyrimethamine resistant strain, shows that if in a situation mefloquine resistance has emerged, cross resistance to quinine may pose a serious problem. It is interesting to point out that both the mefloquine and mefloquine-pyrimethamine resistant strains used in our study are fully sensitive to the action of long acting sulfa drug like dapsone.

Mefloquine resistance in both of our strains discussed above, is labile and the parasites revert to sensitivity after drug-free passage for 70 days. Thus mefloquine-pyrimethamine resistant strain if maintained without mefloquine and pyrimethamine drug pressures, gradually loses mefloquine resistance although it maintains pyrimethamine resistance which is relatively stable. Once the mefloquine resistance is lost the strain acquires sensitivity to quinine as well as 4-aminoquinolines.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Nitya Nand, Director, CDRI, Lucknow, for continued support and encouragement. The supply of gift sample of mefloquine through the courtesy of M/s. Roche Products Ltd., Basel is acknowledged.

REFERENCES

1. Agarwal, K., S.K. Puri and G.P. Dutta (1979). Selection of pyrimethamine resistant strain of *Plasmodium berghei*. *Ind.Jr.Med.Res.*, 69: 577-582.
2. Kazim, M., S.K. Puri and G.P. Dutta (1979). Chemotherapeutic studies with mefloquine and selection of mefloquine resistant strain of *Plasmodium berghei*. *Ind.Jr.Med.Res.*, 70 (suppl.), 95-102.
3. Merkli, B., R. Richle and W. Peters, (1980). The inhibitory effect of drug combination in the development of mefloquine resistance in *Plasmodium berghei*. *Ann.Trop.Med.Parasit.*, 74: 1-9.
4. Peters, W., J. Portus and B.L. Robinson, (1977). The chemotherapy of rodent malaria, XXVIII The development of resistance to mefloquine (WR 142, 490) *Ann.Trop.Med.Parasit.*, 71: 419-427.
5. World Health Organization, 1981. Newsletter, Special programme for research and training in Tropical diseases, No. 16: p.4.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 97-100

Glucose-6 phosphate dehydrogenase in *Plasmodium knowlesi*

INDRESH KUMAR SRIVASTAVA[1], NALINI SAXENA, V.C. PANDEY and G.P. DUTTA[1]

Different cell-free stages (rings, trophozoites, and schizonts) of malarial parasite *P.knowlesi* showed significant activity of G-6-PD. Sequential studies on G-6-PD activity in erythrocytes before and after *P.knowlesi* infection, indicated that enzymes progressively increased with increase in parasitaemia and the maximum increase was two-fold compared to the pre-infection values. The elevated level of enzyme was brought back to normal level after 3 weeks of treatment although parasitaemia was cleared within 72 hrs. of chloroquine treatment.

INTRODUCTION

It is well known that glucose-6-phosphate dehydrogenase (G-6-PD) plays an important role in maintaining the shape and the reduced metabolic state of RBC. It also prevents the erythrocytes from oxidative stress and helps in providing pentoses for the nucleic acid synthesis. (Barnes *et al.*, 1969; Theakston *et al.*, 1976; Sherman, 1979).

Human cases showing G-6-PD deficiency in RBC are highly susceptible to the haemolytic action of primaquine (Gilles and Ikeme, 1960; Madan *et al.*, 1981).

The deficiency of this enzyme in the erythrocytes, has been correlated to the resistance of the RBC to malaria infection probably due to the non-availability of the pentoses which the parasite derives from the host RBC for its nucleic acid synthesis (Allison and Clyde, 1961; Gilles *et al.*, 1967; Theakston *et al.*, 1976; Bienzle *et al.*, 1979; Rao and Goud, 1979).

A marked increase in the enzyme activity in the total homogenate of the erythrocytes of rhesus monkey after *P.knowlesi* infection was observed but the increase was not proportional to the increase in parasitaemia (Fletcher and Maegraith, 1962; Sherman, 1979).

However, several workers have reported the absence of this enzyme in the cell free parasites (Fletcher and Maegraith, 1972; Barnes *et al.*, 1969; Theakston and Fletcher 1973; Theakston *et al.*, 1976; Fletcher *et al.* 1977).

Recent studies on the contrary have demonstrated the presence of this enzyme in the purified merozoites and schizonts of *P.knowlesi* and *P.falciparum* (Haplemann and Wilson, 1981).

The present communication deals with the demonstration of the enzyme G-6-PD in the total homogenate of erythrocytes at different levels of *P.knowlesi* infection, as well as in purified cell free parasites. Further, changes in G-6-PD activity were also studied after curative treatment with chloroquine.

MATERIAL AND METHOD

Adult rhesus monkeys *Macaca mulatta* of either sex weighing about 3-6 kg were inoculated with the strain of *P.knowlesi* (inoculum 10^5) and they were kept under 12 hr photoperiodicity with the fluorescent lights on from 7.00 hour to 19.00 hour.

After infection became patent, the parasitaemia was recorded daily by examining the blood smears stained with Giemsa. For assay of G-6-PD, the blood was drawn from six monkeys before malaria infection and then at different parasitaemia levels after infection. Another group of six monkeys was infected and later given radical curative chloroquine therapy at parasitaemia ranging between 9-28%. Chloroquine was given orally at 20 mg/kg

Accepted for publication, 9 November 1982.
Division of Biochemistry and Microbiology[1],
Central Drug Research Institute,
Lucknow.

dose for 3 days. Blood samples were further drawn serially from drug treated monkeys to see the reversal of G-6-PD to the normal level. For studies on G-6-PD in parasites, the stage of infection was checked by blood smear examination and the monkeys were bled at different parasitaemia levels in acid citrate dextrose (Banyal *et al.* 1979). The blood was centrifuged at 1000 g for 20 minutes, and the plasma and buffy coat were removed. The infected and uninfected erythrocyte homogenate was assayed for G-6-PD. A part of the blood was subjected to the schizont purification. Brown layer of schizonts was aspirated, leaving the uninfected RBC at the bottom. The schizont layer was further purified by ficoll gradient and washed several times to get the purified schizonts for enzyme assay.

Cell free parasite: Monkeys infected with *P.knowlesi* were bled at the different stages of the parasite i.e., rings, trophozoites and schizonts stage. The blood was collected in the citrate saline by the cardiac puncture and centrifuged at 1000 g for 20 minutes. Erythrocytes freed from plasma and buffy coat, were washed three times with chilled saline to remove the leucocytes. The washed erythrocytes were homogenized or lysed for 30 minutes with 0.2% (w/v) saponin in saline. The lysed suspension was then centrifuged at 7,000 g for 30 minutes which yielded three fractions; the upper most layer or haemolysate, followed by ghosts and the parasites were present in the sediment. The parasites were separated from the ghosts by repeated washing with cold normal saline by centrifugation at 7,000 g for 30 minutes and designated as the cell-free parasites.

Preparation of homogenate: Freshly harvested or stored erythrocytes and cell free parasites, were homogenized in Potter-Elvehjem-homogenizer at 4°C in cold glass-distilled water for 15 minutes and used as a source of enzyme.

Assay of glucose-6-phosphate dehydrogenase: (D-glucose-6-phosphate: NADP Oxido reductase, EC 1. 1. 1. 49). G-6-PD activity was demonstrated in different fractions according to the slightly modified method described in World Health Organisation Technical Report series No.366, (1967)

A typical reaction mixture in a final volume of 3.0 ml contained suitably diluted enzyme; Tris HCl-buffer 150μ moles (pH. 7.8), glucose-6-phosphate 20 μ moles, 10 μ moles $MgCl_2$ and NADP 0.27 μ moles. Increase in optical density (O.D.) at 340 nm. was followed for 5 minutes. 3 times for each sample under optimal condition.

Unit: One unit of enzyme activity was that amount of enzyme which reduced one μ mole of NADP/minute/ml of enzyme under our assay system.

Specific activity: Specific activity was expressed as enzyme unit/mg enzyme protein.

Protein estimation: Protein was estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

RESULTS

The data on cell-free parasite showed significantly high G-6-PD activity in trophozoites and schizont stages whereas in ring stage the activity was low (Table 1). Sequential studies on normal erythrocytes (pre-infection) and after infection at increasing level of parasitaemia. (0.04-17.28%), showed a gradual increase of G-6-PD activity (Table 2). At higher level of parasitaemia (9-28%), the G-6-PD activity became nearly double compared to pre-infection (healthy) level. Following chloroquine therapy, (20 mg/kg/day continued for 3 days) the parasitaemia was cleared within 72 hrs. but G-6-PD assay showed, that elevated enzyme level returned to normal level after nearly three weeks (Table 2).

Table—1. Glucose-6-Phosphate dehydrogenase activity in different erythrocytic stages of *P. knowlesi* parasite

Stage of Parasite	Parasitaemia	G-6-PD specific activity
Rings	95%	0.276
Trophozoites	70%	1.77
Schizonts	60%	1.68

Further studies were carried out to compare G-6-PD activity of infected erythrocytes (at 65% parasitaemia), schizont enriched layer, purified schizonts and uninfected cells from the infected blood, which

sediment below the brown layer of schizonts (Table 3). Again the highest activity was found in purified schizonts (1.67 ± 0.002), moderate activity in infected RBC and schizont enriched layer and lowest in uninfected RBC (0.500 ± 0.014). The normal

Table 2. Glucose-6-Phosphate dehydrogenase activity at different level of *P.knowlesi* infection and after drug treatment.

Mean parasitaemia in %	Ranges of parasitaemia	G-6-PD Specific activity (Mean ± S.D.)	Blood drawn after drug administration (No. of days)
0.00	0.00 - 0.00	0.376 ± 0.0275	--
0.04	0.01 - 0.08	0.527 ± 0.0833	
0.60	0.30 - 0.90	0.597 ± 0.0789	
4.52	2.80 - 8.00	0.696 ± 0.0651	
13.28	9.00 -28.00	0.782 ± 0.1570	
After administration of Drug Chloroquine (20 mg/kg)			
6.2	4.2 - 8.00	0.658 ± 0.0980	1
0.88	1.4 - 0.50	0.518 ± 0.1002	3
0.00	0.00 - 0.00	0.514 ± 0.1200	5
0.00	0.00 - 0.00	0.442 ± 0.0930	7
0.00	0.00 - 0.00	0.406 ± 0.0681	14
0.00	0.00 - 0.00	0.397 ± 0.0538	21

Table 3. Glucose-6-phosphate dehydrogenase enzyme in different fractions of *P.knowlesi* infected blood during purification of parasite (Blood was collected at 65% parasitaemia)

Different fractions	G-6-PD Specific activity
Infected RBC	0.978 ± 0.015
Schizont enriched	0.819 ± 0.013
Uninfected RBC	0.500 ± 0.014
Schizonts	1.67 ± 0.002
Normal RBC	0.342 ± 0.035

RBC of healthy monkey showed lowest activity of this enzyme (0.342 ± 0.035).

DISCUSSION

The present study clearly shows the existence of basal level of G-6-PD in rhesus erythrocytes. This level of enzyme is enhanced after the infection of RBC with *P.knowlesi*. Several other workers have also reported the presence of this enzyme in malaria infected RBC of rhesus monkey (Fletcher and Maegraith 1962) and rodents (Langer *et al.*,1967).

Further, the increase of specific enzyme activity was not proportional to the increase of parasitaemia. This is in agreement with the report of Sherman, (1967) and Fletcher and Maegraith (1962). However, the detection of high level of this enzyme in the purified cell free stages of parasite (rings, trophozoites and schizonts) strongly suggests the parasitic origin of this enzyme. This finding is in contrast to the finding of Fletcher and Maegraith (1962) who reported the absence of enzyme activity in cell-free parasite of *P.knowlesi*. Recently, Hempelmann and Wilson (1981) have demonstrated the presence of G-6-PD in purified merozoite and schizonts of *P. knowlesi*, and *P. falciparum*. Langer *et al.* (1967) have also shown the presence of enzyme G-6-PD in cell-free parasite of *P.berghei*. The elevated level of G-6-PD in infected rhesus RBC gradually came down to the normal basal level after total eradication of the parasitaemia. It shows that, atleast partly, the elevated levels of the enzyme in infected RBC were due to increase in the enzyme content of the RBC itself. Since the recovery of the elevated G-6-PD level to normal basal level took nearly 2-3 weeks after clearance of parasitaemia, an assay of this enzyme may help in the assessment of cure.

ACKNOWLEDGEMENT

The authors are thankful to Dr. Nitya Nand, Director, CDRI for continued interest and support. Thanks are also due to the Director-General, Indian Council of Medical Research, and Council of Scientific and Industrial Research, New Delhi for the award of JRF to Mr. I.K. Srivastava and Miss N. Saxena.

REFERENCES

1. Allison, A.C. and D.F. Clyde (1961). Malaria in African children with deficient erythrocytes Glucose-6-phosphate dehydrogenase. *British Medical Journal*, 1, 1346-1348.
2. Banyal, H.S., V.C. Pandey & G.P. Dutta (1979). Sub-cellular fractionation and localisation of marker enzyme of erythrocytic stages of *P.knowlesi*. *Indian Journal of Parasitology*, 3: 9-14.

3. Barnes, G.B., Herman Potel, Thomas R. Denison and Charles, F. Barr (1969). The influence of methylene blue on the pentose phosphate pathway in erythrocytes of monkeys infected with *P.knowlesi. Journal of Lab. and Clinical Medicine,* **74**: 1-11.

4. Bienzle, U.J. Guggenmoos, Holzmann & L. Luzatto, (1979). Malaria and erythrocyte glucose-6-phosphate dehydrogenase variant in West Africa. *Am.J.Trop.Med.Hyg.*, **28**: 619-621.

5. Fletcher, K.A. and B.G. Maegraith (1962). Glucose - 6 - phosphate dehydrogenase activity in erythrocytes of monkeys infected with *P.knowlesi. Nature,* **196**: 1316-1318.

6. Fletcher, K.A. and B.G. Maegraith, (1972). The metabolism of the malaria parasite and its host. *Adv.Parasitol.* **10**: 31-48.

7. Fletcher, K.A., M.V.Canning and R.G.D. Theakston, (1977). Electrophoresis of glucose-6-phosphate dehydrogenase and 6 phosphogluconate dehydrogenase in erythrocytes from malaria infected animals. *Ann.Trop.Med.Parasit.* **71**: 125-130.

8. Gilles, H.M. and A.C. Ikeme (1960). Haemoglobinuria among adult Nigerians due to G-6-PD deficiency with drug sensitivity. *Lancet,* II: 889-891.

9. Gilles, H.M ; K.A. Fletcher, R.G. Hendrickss, R. Linder; S. Reddy, N. Allen (1967). Glucose-6-phosphate dehydrogenase deficiency sickling and malaria in African children in South Western Nigeria. *Lancet,* **1**: 138-140.

10. Hempelmann and R.J.M. Wilson (1981). Detection of glucose -6-phosphate dehydrogenase in malaria parasite. *Molecular and Biochemical Parasitology,* **2**: (3/4), 197-204.

11. Langer, W., Bernhardt, Piron Phisphumvidhi and Yael Friendlander (1967). Malarial parasite metabolism. The pentose cycle in *P.berghei. Experimental Parasitology,* **20**: 68-76.

12. Lowry, D.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with Folin Phenol reagent. *J. Biol.Chem.* **193**: 265-275.

13. Madan, Nishi, Nerra Talwar, Abha Maheswari and S.K. Sood, (1981). Incidence of G-6-PD. Deficiency in a hospital population of Delhi *Indian Journal of Medical Research,* **73(3)**: 425-429.

14. Rao, P.R. and J.P. Goud (1979). Sickle cell haemoglobin and glucose-6-phosphate dehydrogenase deficiency in tribal population of Andhra Pradesh. *Indian Journal of Medical Research,* **70**: (11), 807-813.

15. Sherman, I.W. (1967) Glucose-6-phosphate dehydrogenase and reduced glutathione in malaria infected erythrocytes (*P.berghei & P.lophurae*) *Journal of Protozoology,* **12**: 394-396.

16. Sherman, I.W. (1979). Biochemistry of *Plasmodium* (Malaria parasite). *Microbiological Reviews,* 43 (4), 453-495.

17. Theakston, R.G.D. and K.A. Fletcher (1973). A technique for the cytochemical demonstration in electron microscope of Glucose-6-phosphate dehydrogenase activity in erythrocytes of malaria infected animals. *J. Microsc* (Oxford) **97**: 315-320.

18. Theakston, R.G.D., K.A. Fletcher and G.A. Moore (1976). Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in human erythrocytes infected with *P. falciparum. Ann.Trop.Med.Parasit.,* **70**: 125-127.

19. World Health Organisation, Technical Report Series No.366 (1967). Standardization of procedure for the study of Glucose-6-phosphate dehydrogenase.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 101-108

Multiple Invasion of Erythrocytes by *Plasmodium vivax*— A Report of 56 Cases

D.S. CHOUDHURY[1], S.K. GHOSH[1] and C. USHA DEVI[1]

Peripheral blood smears collected from patients suffering from malaria belonging to Sonepat district of Haryana, Delhi and Terai region of Uttar Pradesh near Rudrapur showed multiple invasion of erythrocytes by *P. vivax* parasites in 56 of the 2963 slides examined. A total of 19 of these patients had a maximum of four parasites in one erythrocyte. A maximum of eight parasites in one erythrocyte was seen in two patients.

INTRODUCTION

Multiple invasion of erythrocyte is characteristic of *P.falciparum* parasites. Erythrocyte having 2 or 3 parasites is commonly seen in human infection with this species. Springall (1943) reported eight rings in a single erythrocyte in a case having high parasitaemia of *P.falciparum*. In *in-vitro* culture of *P.falciparum* carried out at Malaria Research Centre, the authors found a maximum of ten rings in one erythrocyte. Multiple invasion of erythrocytes is uncommon in infection with *P.vivax* and is very rarely seen in *P.malariae*.

Grassi (1920) reported a single erythrocyte containing a gametocyte and a schizont of *P.vivax* and postulated the theory of parthenogenesis on this finding. Wenyon (1926) refuted this and demonstrated that the particular instance was a case of multiple infection. Field (1942) reported a case with heavy infection of *P.vivax* where one erythrocyte had 8 young trophozoites.

A total of 56 cases of multiple invasion of erythrocytes by *P.vivax* parasites was detected during the investigation of an outbreak of malaria in Sonepat district of Haryana State, Delhi and Terai region of Uttar Pradesh during January 1981-July 1982. The results of the examination of the slides drawn from these cases have been presented in this paper.

Accepted for publication: 16 November 1982.
[1] Malaria Research Centre (ICMR),
22 Sham Nath Marg,
Delhi-110054.

MATERIAL AND METHODS

The patients belong to different villages of Kharkhoda and Halalpur PHCs of Sonepat district of Haryana, Delhi proper and villages situated in Terai region of Uttar Pradesh near Rudrapur. Thin blood smears were prepared from these patients during febrile attacks of malaria. These were stained with J.S.B. stain and examined under oil immersion lens. The results of examination of one thousand parasitized erythrocytes from each smear taken at random were recorded.

RESULTS

A total of 2963 *P.vivax* slides was collected during this investigation. Many of them showed two parasites in a single erythrocyte. Those showing three or more parasites in an erythrocyte were only included in this paper. The data obtained from the examination of these smears are presented in Table 1 and Figs. 1-9. The parasitaemia varied from 0.1 to 4.8 per cent. The patients belonged to different age groups. The youngest was one year old and the oldest sixty-one. Different stages of the parasites from rings to schizonts and gametocytes were seen in the multiple infections. It was observed that a maximum of four parasites of advanced stages from late trophozoites to schizonts and gametocytes were present in these erythrocytes having multiple infection. Some of the young trophozoites (ring form) showed multiple chromatins in the form of double chromatin of equal or unequal size as seen in *P. falciparum* infection. A small accessory chromatin was occasionally seen. 'Tenue' forms of early tro-

Table 1 - Multiple invasion of erythrocytes by five or more parasites of *P. vivax*

Sl. No.	Age in years	Place of collection	Multiple invasion per 1000 parasitized erythrocytes								Parasitaemia (%)
			Erythrocytes having single parasite (%)	Erythrocytes with two parasites (%)	Erythrocytes with three parasites (%)	Erythrocytes with four parasites (%)	Erythrocytes with five parasites (%)	Erythrocytes with six parasites (%)	Erythrocytes with seven parasites (%)	Erythrocytes with eight parasites (%)	
1.	4	Delhi	76.5	19.3	1.6	2.1	0.5			—	0.7
2.	10	"	65.1	29.7	3.8	0.8	0.3	0.3	—	—	2.8
3.	12	"	87.0	10.5	2.1	0.2	0.2	—	—	—	2.2
4.	18	"	42.9	41.6	10.9	3.1	0.9	0.3	0.2	0.1	1.2
5.	12	Sonepat (Haryana)	73.0	22.0	3.6	1.2	0.1	0.1	—	—	1.2
6.	6	U.P. Terai	78.5	17.6	2.8	1.0	0.1	—	—	—	2.1
7.	7	"	82.7	13.8	3.2	0.2	0.1	—	—	—	2.6
8.	9	"	65.8	23.3	5.4	2.9	1.5	0.6	0.2	0.3	3.8
9.	20	"	76.2	18.8	3.3	1.5	0.2	—	—	—	1.2

Out of the remaining 47, 19 had a maximum of four, 28 had a maximum of three parasites in one erythrocyte. They have not been included in this table.

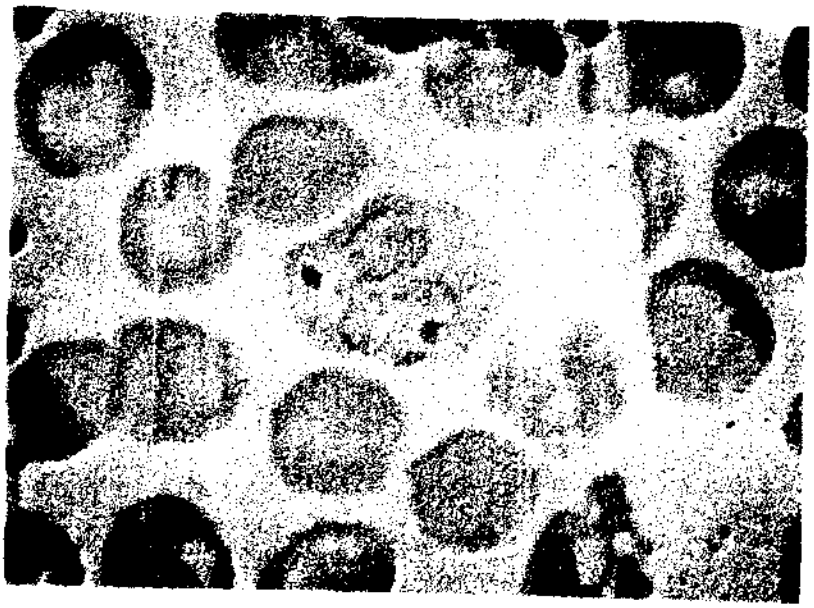


Fig. 1 Showing two trophozoites. (×2000)

phozoites were present in some smears. The erythrocytes with Schuffner's dots in parasitized cells containing the older stages of the parasites were observed to be considerably enlarged. The older trophozoites were highly amoeboid and showed fine golden brown pigments. The schizonts filled the whole of the enlarged erythrocytes and a maximum of four mature schizonts could be seen in these erythrocytes. The number of schizonts in these multiple infections could be ascertained from the collection of large masses of pigments of each parasite. The number of merozoites in each schizont varied from 12 to 20. In some of the slides, two mature gametocytes could be seen in these erythrocytes. Invasions upto a maximum of eight early trophozoites could be seen in erythrocytes of two patients aged 9 and 18 years respectively. Two others had a maximum of six parasites in an erythrocyte. Their ages were 10 and 12 years respectively. Five cases had a maximum of five parasites in one erythrocyte. Ninteen had a maximum of four and twenty-eight of three parasites per erythrocyte respectively.

DISCUSSION

Das Gupta (1939) reported multiple infection of *P.vivax* in an infant 15 hrs. after birth. Five parasites in a single erythrocyte were found in this patient. Some of the ring stages had two chromatin dots. None of the mature schizonts had more than twelve merozoites. Field *et al.* (1939) also reported multiple infection of *P.vivax* in Malaya in a 16 day old infant. Multiple infections upto three parasites in a single erythrocyte were seen. Since both the cases were in very young infants, the possibility of the host factor playing some role was suggested. In the present study, the patients with multiple parasites in an erythrocyte belonged to different age groups. A maximum of eight parasites in a single erythrocyte was seen in a 9 year old boy and also in a 18 year old girl.

Wang (1970) reported multiple infection by two parasites in a single erythrocyte in three patients suffering from *P.vivax* malaria and concluded that in high parasitaemia multiple invasions take place.

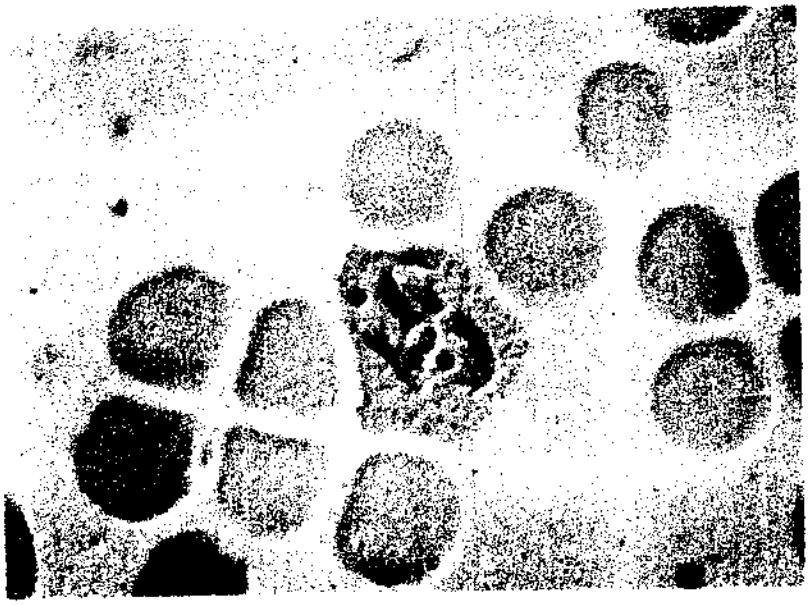


Fig. 2 Showing three trophozoites.
(×2000)

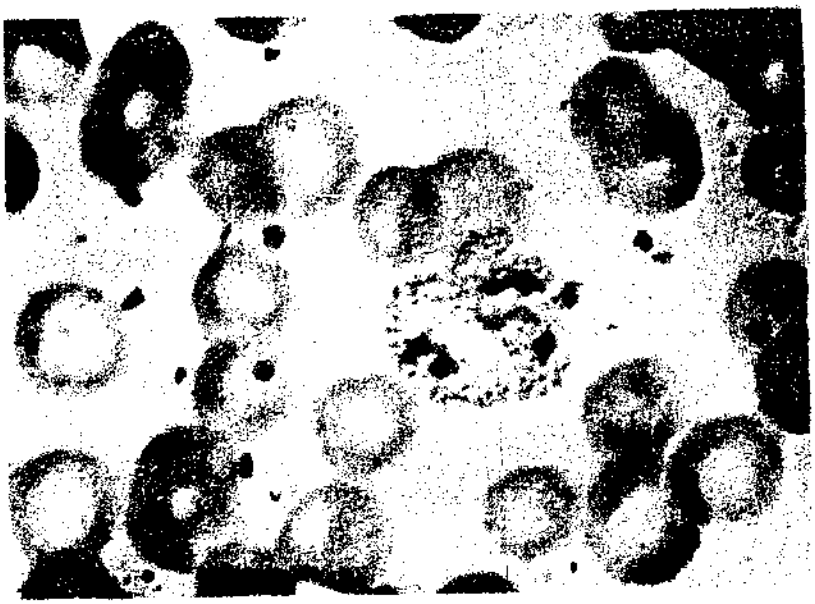


Fig. 3 Showing four trophozoites.
(×2000)

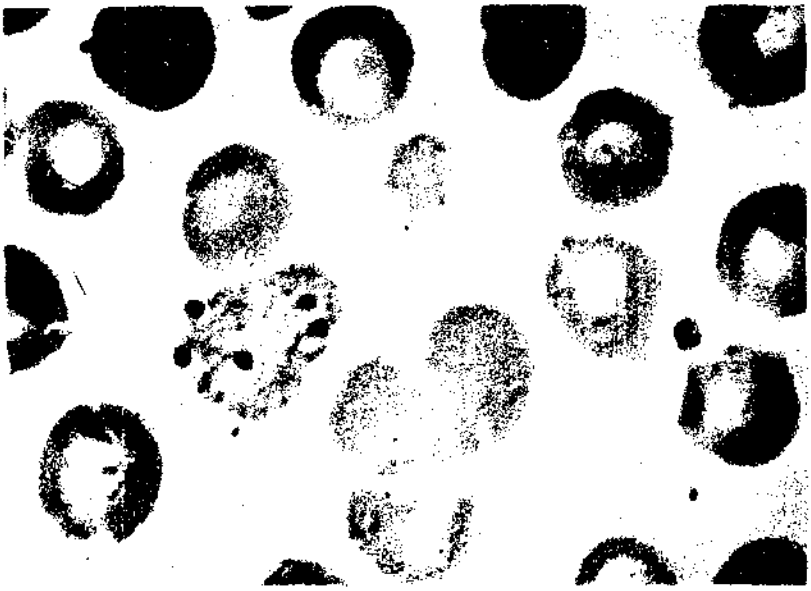


Fig. 4 Showing five trophozoites.
(×2000)

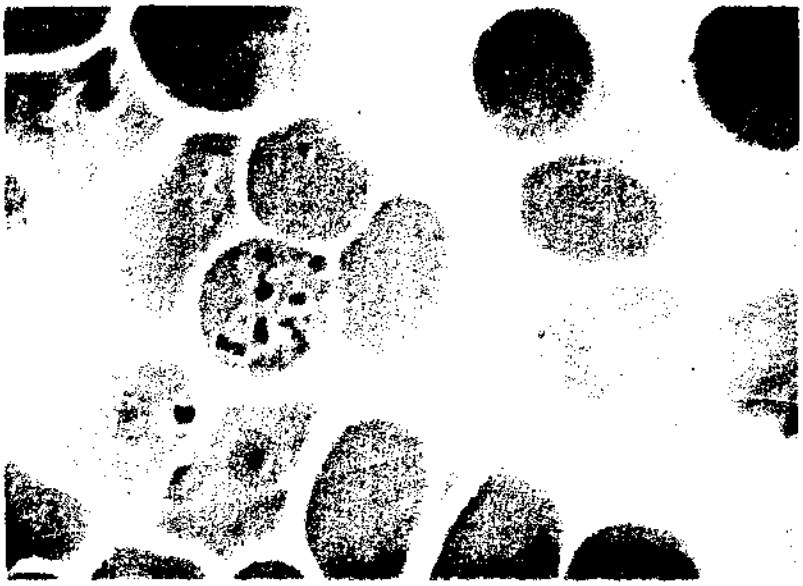


Fig. 5 Showing six trophozoites
(×2000)

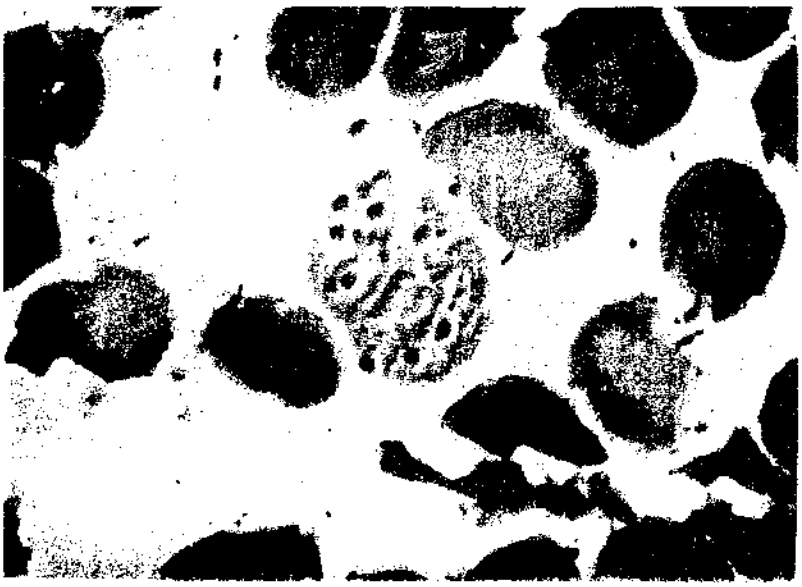


Fig. 6 Showing eight trophozoites.
(×2000)

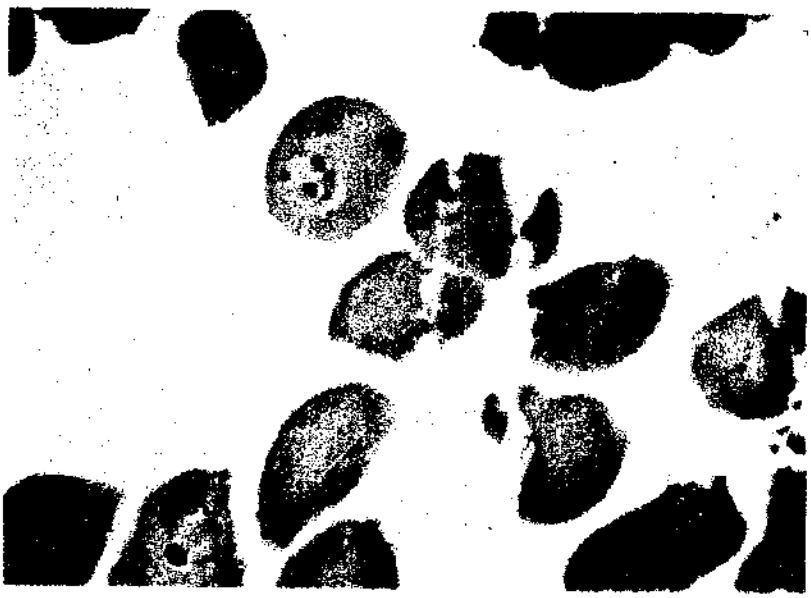


Fig. 7 Showing accessory chromatin
(×2000)

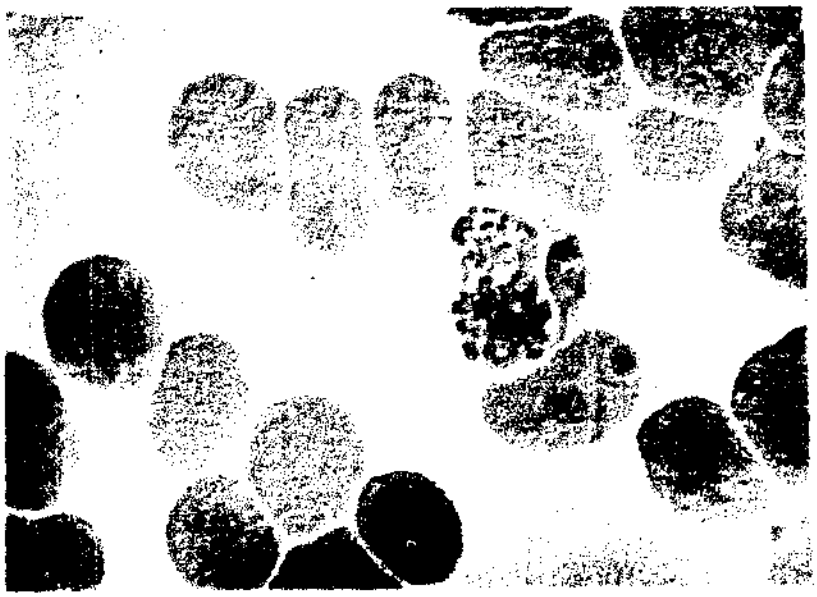


Fig. 8 — Showing a schizont, a gametocyte and a trophozoite.
(× 2000)

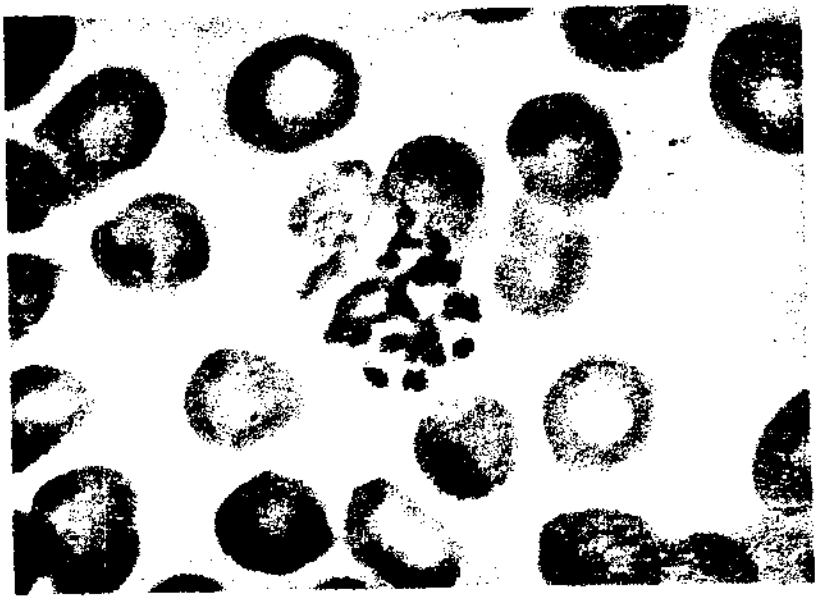


Fig. 9 — Showing two schizonts and one gametocyte
(× 2000)

In the present series, two patients having 3.8 per cent and 1.2 per cent parasitaemia respectively showed multiple infection upto eight parasites. Two other patients who showed a maximum of six parasites in an erythrocyte had parasitaemia of 1.2 and 2.8 per cent respectively. In the 19 patients having a maximum of four parasites, the parasitaemia varied from 0.4 to 3.4 per cent.

Tsukamoto (1977) reported a case of a Japanese student suffering from *P. vivax* like malaria in which heavy multiple infections of the erythrocytes were seen. The parasitaemia in this case was only 0.82 per cent. More than 30 per cent of the infected erythrocytes showed multiple infections of two or more young trophozoites. A maximum of 8 rings were seen in these erythrocytes. The schizonts had twelve to nineteen merozoites.

As mentioned earlier, multiple infection is not common in *P. vivax* infection. The inability of the erythrocyte to harbour more than one parasite without rupturing may be responsible for this. It seems that the strain of *P. vivax* seen in the cases reported here is likely to be different from the normal classical *P. vivax* strain found in India. In this Jet age, importation of strains has to be kept in mind. Some of these strains seem to possess some characteristics of *P. falciparum*. Further investigations regarding the extent of prevalence of the strains and other characteristics like susceptibility to antimalarials, the relapse pattern etc., need to be carried out to establish their identity.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. V.P. Sharma, Director, Malaria Research Centre for his help in the investigations. The technical assistance received from Sh. M.I.H. Usmani, Samuel Senaputra, P.R. Chawla and Satish Kumar in the collection of the slides is gratefully acknowledged.

REFERENCES

1. Das Gupta, B.M. (1939). Some anomalies in the morphology of *Plasmodium vivax* occurring in a new born baby. *Ind. Med. Gaz.* **74(5)**: 273-274.
2. Field, J.W. & J.C. Niven. (1939). Anomalies in the morphology of *Plasmodium vivax*. Letter to the *Ind. Med. Gaz.*, **74(8)**: 509.
3. Field, J.W. (1942). Morphological variation in *Plasmodium vivax* Grassi and Feletti, 1890. *Parasitology* **34**: 82-87.
*4. Grassi, B. (1920). Studi di un zoologo sulla Malaria, Rome.
5. Springall, A.N. (1943). Heavy density of *Plasmodium falciparum* parasites in malaria. Report of a case. *Am. J. Trop. Med.*, **23(5)**: 533-535.
6. Tsukamoto, M. (1977). An imported human malarial case characterized by severe multiple infections of the red blood cells. *Trop. Med.* **19(2)**: 95-104.
7. Wang, C.C. (1970). Multiple invasion of erythrocyte by malaria parasites. *Tran. Roy. Soc. Trop. Med. Hyg.*, **64(2)**: 268-270.
8. Wenyon, C.M. (1926). Protozoology. Vol. II. Bailliere Tindall & Cox. London, P-933.

*Original paper could not be consulted.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 109-114

Glutathione Metabolism of *Plasmodium berghei* Parasitized Erythrocytes in Mice

R.K. SETH[1], A.S. SAINI[1], S.K. AGGARWAL[1]

Glutathione metabolism was studied in infected and non-infected erythrocytes in 31 mice infected with *Plasmodium berghei*. The efficiency of glutathione metabolism in *Plasmodium berghei* infected red cells was found to be increased, as parasite adds to the activity of some of the enzymes of this protective mechanism. This is an effort on the part of the parasite to protect the environment in which it is growing.

INTRODUCTION

Haemolytic anaemia is one of the serious complications of malaria. The pathogenesis of anaemia associated with malaria remains obscure despite a great deal of work (Weatherall *et al.*, 1982). Although much emphasis has been laid upon autoimmune mechanism (Zuckerman 1964) as a cause of excessive haemolysis but relationship between decreased red cell survival as a result of increased oxidant stress on red blood cell is least understood, as parasite is known to generate oxidants (Etkin *et al.*, 1975). However the protection of erythrocyte from this oxidant damage is brought about by oxidant removing glutathion system (Fig. 1) (Leipzig *et al.*, 1975). It is therefore apparent that inefficiency of this glutathione system may make the red cell more vulnerable to oxidant damage.

We have, therefore, conducted a study of glutathione metabolism in parasitized and nonparasitized erythrocytes in mice infected with *P.berghei*. The study is simultaneously conducted in the erythrocytes of 10 control animals.

MATERIAL AND METHODS

Young Swiss white mice were inoculated intraperitoneally with parasitized mouse blood diluted in phosphate buffered saline (PBS). The mice received an average dose of one million parasites. The parasites usually appeared in peripheral blood on 3rd day following inoculation. Giemsa-stained blood smears were obtained daily to follow the progress of infection until parasitemia reached the peak level (70% or slightly more) in 7-10 days following inoculation. It is noteworthy that parasitemia rarely crossed 70% at which level most of the mice died. At various degrees of parasitemia blood from infected mice was collected, after ether anaesthesia by cardiac puncture and aspiration into EDTA solution at 4°C. Leucocytes were removed by filtration through micro cellulose columns (Fulton *et al.*, 1956) and parasitized erythrocytes were separated from non parasitized ones on ficoll cushion (Eling 1977). Separation achieved by this gradient was 100% at low levels of parasitemia (upto 50%) but blood having high degree of parasitemia (50%-70% or more) required broad centrifuge tubes with large surface area for centrifugation to achieve the same degree of purity.

Although, increase in reticulocyte count was observed after 5th day of plasmodial infection, this increase was not statistically significant.

Haemoglobin estimation in lysates were made by cyanmethaemoglobin method (Dacie and Lewis 1975), to know the progress of anaemia. Erythrocyte count was done in Neubauer-chamber. Erythrocyte glutathione (GSH) was measured with alloxan (Kay *et al.*, 1960).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Lohr *et al.*, 1974), Glutathione reductase (EC

Accepted for publication, 22 December 1982.
[1] Department of Biochemistry,
Medical College,
Rohtak-124001.

1.6.4.2.) (Beutler 1975) and Glutathione peroxidase (EC 1.11.1.9.) (Hopkins *et al.*, 1973) were measured spectrophotometrically.

RESULTS

A. *Reduced Glutathione* (GSH): A constant increase in GSH contents was observed in parasitized erythrocytes through group I ($p<0.05$) to group IV ($p<0.001$) Table I. However, no significant increase in total GSH content was found in non-parasitized erythrocytes ($p>0.05$).

B. Glucose-6-phosphate dehydrogenase (G-6-PD): There was no significant elevation in G-6-PD levels in parasitized and non parasitized erythrocytes ($p>0.05$) but parasitized red cells had a decreasing trend in comparison with the normal erythrocytes (Table I).

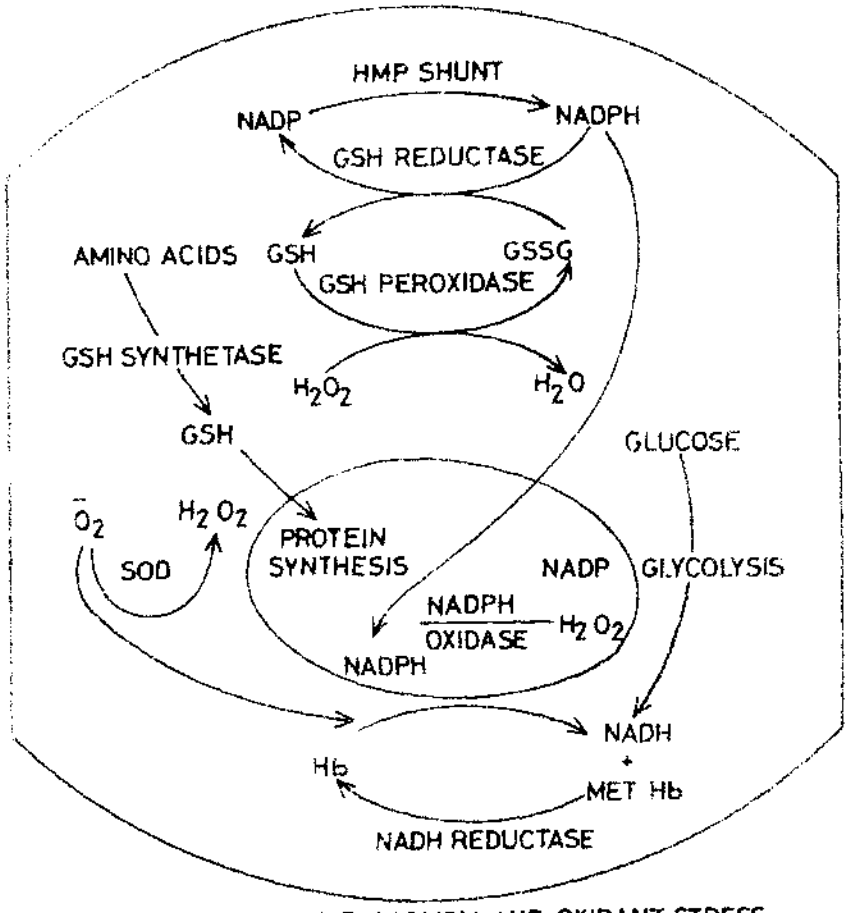


Fig. 1—The utilization of reducing equivalents (Viz GSH and NADPH) and protection of *P. berghei* infected red blood cells in mice.

Table 1. Reduced-glutathione (GSH) contents, and the activity of Glucose-6-phospinate dehydrogenase (G-6-PD) Glutathione Reductase (GR) and Glutathione peroxidase (GSH-Px) in control infected and noninfected erythrocytes (values are mean ± SD).

		GSH (mg. 10^{10} RBC's)	G6PD (IU/10^{10} RBC's)	GR (IU/10^{10} RBC's)	GSH-Px (IU/10^{10} RBC's)
	C Control (n=10)	1.28 ± 0.34	3.36 ± 0.62	4.03 ± 1.03	12.6 ± 1.47
Group -I (n=6) (15%-30% erythrocytes infected)	A --Infected	1.74 ± 0.28*	3.55 ± 0.40	6.16 ± 1.1*	17.2 ± 2.18*
	B --Noninfected	1.66 ± 0.20	3.71 ± 0.54	4.64 ± 0.73	14.12 ± 2.88
Group -II (n=8) (30%-50% Erythrocytes infected)	A --Infected	1.95 ± 0.38*	3.09 ± 0.51	7.85 ± 0.57*	19.82 ± 1.57
	B --Noninfected	1.36 ± 0.22	3.47 ± 0.48	4.26 ± 0.77	16.62 ± 2.3*
Group III (n=10) (50%-70% Erythrocytes infected)	A --Infected	2.10 ± 0.47*	3.26 ± 0.07	7.07 ± 0.83*	18.12 ± 2.08*
	B--Noninfected	1.41 ± 0.39	3.74 ± 0.36	4.19 ± 0.72	17.54 ± 3.51*
Group IV (n=7) (70% Erythrocytes infected)	A --Infected	2.15 ± 0.50*	2.61 ± 0.78	6.4 ± 1.19*	20.99 ± 3.37*
	B --Noninfected	1.48 ± 0.40	3.32 ± 0.40	4.6 ± 0.65	16.37 ± 3.07*
Total (n=31) (15%-70% Erythrocytes infected)	A --Infected	2.0 ± 0.42*	3.06 ± 0.64	6.91 ± 1.28*	18.12 ± 2.08*
	B Noninfected	1.45 ± 0.33	3.61 ± 0.48	4.37 ± 0.70	16.33 ± 3.11*

Values of infected and non infected red cells are compared with the values of control (A Vs C and B Vs C) -(Student's *t* test-independent)

* Significant

For comparison of infected Vs noninfected red cells (A Vs B)---(Student's *t* test paired) and level of significance (p value) sex text

C. Glutathione Reductase: In group II and III the activity of GR was tremendously increased (Table I) in comparison with the normal control group ($p<0.001$) though an increase in the activity of GR at all levels of parasitemia was observed. Non-parasitized red cells did not show any increase in the activity of GR ($p>0.05$).

D. Glutathione Peroxidase (GSH-Px): There is a dramatic increase in GSH-Px activity in parasitized erythrocytes with the onset of malarial infestation in animals ($p<0.001$). Non parasitized erythrocytes also showed a significant increase in the activity of this enzyme as compared with those of control ($p<0.001$)

DISCUSSION

The malarial parasite has a metabolic unity with the host erythrocyte from which it is demarcated by the parasite cell membrane. The composition of the parasite and the permeability of parasite membrane inside the erythrocyte are very little understood. However, parasite generates oxidants (Etkin *et al.*, 1975). Reduced glutathione (GSH) protects the erythrocyte from this oxidant damage.

A markedly increased reflection of GSH in parasitized erythrocytes (Group II through Group IV, Table I) is in agreement with the findings of the previous workers (Picard-Maureau *et al.*, 1975). They observed an increase in the activity of already existing GSH-synthetase in *P.vinckei* parasitized erythrocytes. Presumably, the reason for the increased level of GSH in parasitized erythrocytes is partly due to the increased activity of glutathione reductase (Table I, Fig. 1). Since parasite has been reported to synthesize its own enzyme (Eckman *et al.*, 1979). Also a close correlation is known to exist between the level of erythrocyte GSH and the hexose mono phosphate (HMP) shunt enzymes (Fig. 1) and the tendency to hemolysis. But there seems to be a decreasing trend rather than increase in the activity of first enzyme of HMP shunt, G-6-PD, in parasitized erythrocytes (Table I), though this decrease is not statistically significant. Therefore NADPH requirement for parasite glutathione-reductase is accomplished from a pathway other than HMP shunt since G-6-PD is absent from all malarial parasites studied (Sherman 1979). However glutamate dehydrogenase may be the source of additional requirement of NADPH in *P.berghei* malaria (Langer *et al.*, 1970).

We have found a dramatic increase in the activity of glutathione peroxidase (GSH-Px) in parasitized erythrocytes (Table I). Like glutathione reductase, this enzyme may also originate from parasite itself. The possible activation of this enzyme may be due to an activator or the removal of the inhibitor from the infected erythrocytes. Therefore we propose that the increase in the activity of these two enzymes (viz. Glutathione reductase and Glutathione peroxidase) of glutathione cascade within the infected erythrocytes might be related to an effort on the part of the parasite to protect the integrity of the environment in which it is growing; since hydrogen peroxide production is a metabolic feature of parasite itself (Etkin *et al.*, 1975, Friedman, 1979).

Increase in the activity of glutathione peroxidase in unparasitized erythrocytes, seen in the present study, is difficult to explain. There is every likelihood of the possibility that the newly synthesized erythrocytes from erythroid precursors might be the source of this enzyme. This is currently under investigation in our laboratory.

ACKNOWLEDGEMENTS

The generous financial assistance provided by I.C.M.R. (Scheme No.5/3-2(4)-1980) New Delhi is highly acknowledged. We extend our sincere thanks to Dr. D. Subrahmanyam (Director, CIBA Research Centre, Bombay) and Dr. (Mrs.) S. Sehgal, Professor of Immunopathology P.G.I. Chandigarh for providing malaria strain without which this work would have been impossible.

REFERENCES

1. Beutler, E. (1975). Red cell metabolism - a manual of biochemical methods (Grune and Stratton Publishers, New York).
2. Dacie, J.V. and S.M. Lewis (1975). Practical haematology (The English language book society and Churchill Livingstone).
3. Eckman, J.R. and J.W. Eaton (1979). Dependence of plasmodial glutathione metabolism on the host cell. *Nature (London)*, **222**: 389.
4. Eling, W. (1977) Ficoll fractionation for the separation of

parasitized erythrocytes from malaria infected blood. *Bull. WHO* **55**: 105.

5. Eaton, N.L. and J.W. Eaton (1975). Malaria induced erythrocyte oxidant sensitivity. In Erythrocyte structure and function: (Brewer, G.J. ed.) Vol. I, pp. 219-234. (Elsevier Pub. Co. New York).

6. Friedman, M.J. (1979). Oxidant damage mediates variant red cell resistance to malaria. *Nature (London)*, **280**: 245-247.

7. Fulton, J.D. and P.T. Grant (1956). The sulfur requirements of the erythrocytic form of *P.knowlesi*. *Biochem. J.* **63**: 274.

8. Hopkins, J. and G.R. Tudhope (1973). Glutathione peroxidase in human red cells in health and disease. *Brit. J. Haematol.* **25**: 563-567.

9. Kay, W.M. and K.C. Murlin (1960). The determination of blood glutathione. *Biochem. J.* **74**: 203.

10. Langer, R.W., Jr., P. Phisphumvidh and D. Jampermpoon. (1970). Malarial parasite metabolism: the glutamic acid dehydrogenase of plasmodium berghei. *Exp. Parasitol.* **28**: 298-303.

11. Leipzig, R.M., G.J. Brewer and W.C. Kruckerberg. (1975). Superoxide and G-6-PD type hemolysis. In "Erythrocyte structure and function" Vol. I (Brewer, G.J. ed.). Elsevier scientific Pub. Co. New York).

12. Lohr, G.W. and H.D. Waller (1974). Methods of enzymatic analysis Vol. 2 (Bergmeyer H.U. ed.) Academic Press Inc. New York)

13. Picard-Maureau, A., E. Hempelmann, G. Krammer, R. Jackisch and A. Jung (1975). Glutathione status in Plasmodium vinckei parasitierten erythrozyten in abhangigkeit vom intraerythrozytaren entwicklungsstadium des parasiten. *Tropenmed parasit.* **26**: 405-416.

14. Sherman, I.W. (1979). Biochemistry of plasmodium (malarial parasites). *Microbiol. Rev.* **43**: 453-495.

15. Weatherall, D.J. and S. Abdalla (1982). The anaemia of *Plasmodium falciparum* malaria. *Brit. Med. Bull.* **38**: 147-151.

16. Zuckerman, A. (1964). Autoimmunization and other types of indirect damage to host cells as factors in certain protozoan diseases. *Exp. Parasitol.* **15**: 138-183.

Indian Journal of Malariology
Vol. 29, Dec 1982, pp 115-129

The Effect of Immunostimulants in Modulating the Behaviour of *P. berghei*

[illegible]

Various immune adjuvants were administered to rats and mice to assess their relative roles in modulating *P.berghei* infection. The study indicates that BCG, Corynebacterium parvum and complete Freund's adjuvant can protect the inbred rats and mice irrespective of the parasite load. A single dose of 0.2 mg BCG administered intravenously with the parasite afforded a complete protection in mice. Contrary to earlier published series, subcutaneous injections of a mixture of BCG and parasites were equally effective and even ICRC strain of mice could be completely protected by this manuover. Protection observed by *C.parvum* was a time bound phenomenon and faded after a time. BCG appears to be the most effective adjuvant in the rodent model and unlike complete Freund's adjuvant is devoid of local deleterious effects.

INTRODUCTION

From time to time several devices have been adopted to stimulate the putative surveillance mechanisms of the host. Convincingly enough, under certain circumstances these manoeuvres have established control of transplantable tumors, bacterial infections, protozoal infections etc. (Zbar *et al.*, 1971). Considering the vast tropical world affected by parasitic diseases the role of immunostimulation by adjuvants cannot be over emphasized.

The resistance to these organisms can be achieved by potentiating the effector pathways of specific immunity. Several natural and synthetic compounds including thymic hormones, bestatine, tuftsin, levamisole, BCG etc. have been used with interesting but varying results (Dukor *et al.*, 1979). Our earlier observations (Parashar *et al.*, 1977) on cell transfer studies indicated that frozen thawed macrophages were capable of affording a remarkable degree of protection and it was postulated that immune macrophages may be able to limit the parasitaemia by processing the protective antigens. Further, we were also able to process these antigens *in vitro* (Parashar *et al.*, 1982). In the present study an attempt was made to stimulate the Mononuclear Phagocyte System (MPS) using BCG, C. parvum and Complete Freund's Adjuvant (CFA) to assess their relative roles in altering the course of parasitaemia.

MATERIAL AND METHODS

Animals: Both young rats (Wistar strain) weighing 70-90 gms; and ICRC Swiss inbred mice weighing 20-25 gms. were used in this study. The animals were kept on pellet diet (Hindustan Levers) and water *ad libitum*.

Parasite: *P.berghei* (Vincke & Lips 1948) obtained from National Institute of Communicable Diseases, Delhi, has been weekly *passaged* in the laboratory using infected red blood cells with 1×10^7 (IRBCs) as standard inoculum for rats and 1×10^6 IRBCs for mice by the intraperitoneal route (Parashar *et al.*, 1977).

Design of Experiments: BCG was obtained from Madras Tuberculosis Research Centre, India as 5 mg dry wt. ampoules. The contents of the ampoule were dissolved in 5 ml of sterile saline and 0.2 ml/rat or 0.1 ml/mice i.p. were given alongwith the parasites or separately in 3 doses at 21 days interval. Since *P.berghei* is a self limiting infection in rats, multiple parasite challenges were used in order to assess the degree of protection. Similar experiments were conducted using Complete Freund's Adjuvant (obtained from Difco Labs., Michigan USA) in the

Accepted for publication 23 December 1982.
[1]Department of Immunopathology,
Postgraduate Institute of Medical Education and Research, Chandigarh-160012

dosage of 0.2 ml rat S.C. or 0.1 ml mouse S.C. in different groups of animals. Corynebacterium parvum was obtained from Burough's Wellcome, England as a formalin killed suspension (7 mg dry wt/ml). Daily smears were made to check parasitaemia. From all the groups serial blood samples were collected and tested for parasitaemia and for the presence of Ab. and Ag. by counter current immuno-electrophoresis (CIEP).

Experiment I (Figure 1 and 1a). *Out bred rats*

Alternate immunostimulant, parasite schedule

- Control
- BCG three doses on 0, 21, 42 days
- CFA three doses on 0, 21, 42 days
- C.parvum .2 ml on 0, 21, 42 days

All test animals receiving parasite challenges on 10, 20, 30, 40 and 50 days.

Experiment II (Figure 2)

priming with *adjuvant parasite mixture in four doses* on 0, 21, 42, 63 days and infection on day 72.

1. BCG + P* I.V.
2. BCG + P* I.M.
3. Parasite S.C.
4. Parasite I.M.
5. Control

* Parasite number sequentially increasing from 10^5-10^6

Experiment III in *inbred rats* (Figure 3)

Groups

1. BCG + Parasite S.C. separately
2. BCG + Parasite IV after incubation
3. CFA + Parasite S.C.
4. Parasite alone S.C.
5. Parasite alone I.P.

Three doses given at 0, 21, 42 days followed by parasite challenge on 49th day.

Design of experiment IV in ICRC mice:

Three doses of each on 0, 21, 42 days

1. BCG alone S.C.
2. BCG + Parasite S.C. separately
3. BCG + Parasite S.C. in a mixture
4. BCG + Parasite I.V.

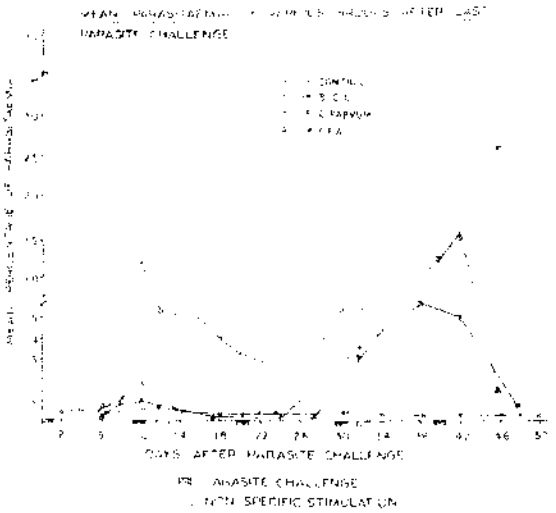


Fig. 1 Graph showing complete protection by CFA and significant protection with BCG and C.parvum in out bred rats.

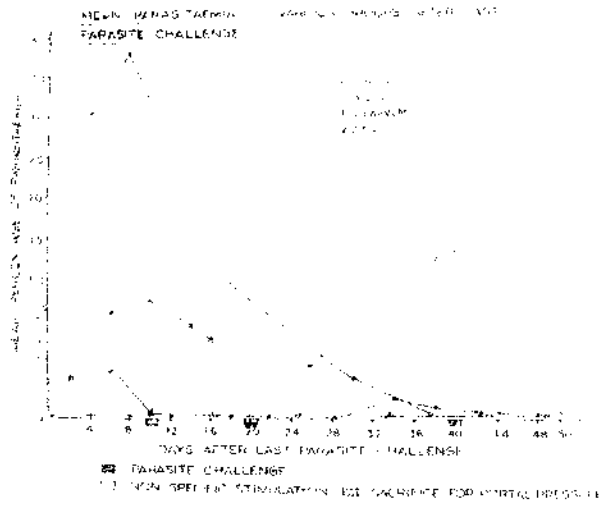


Fig. 1a Confirmation of curves in Fig. 1
Long term follow up of same animals as in Fig. 1. Protection induced by C.parvum waved off after 80 days of observation.

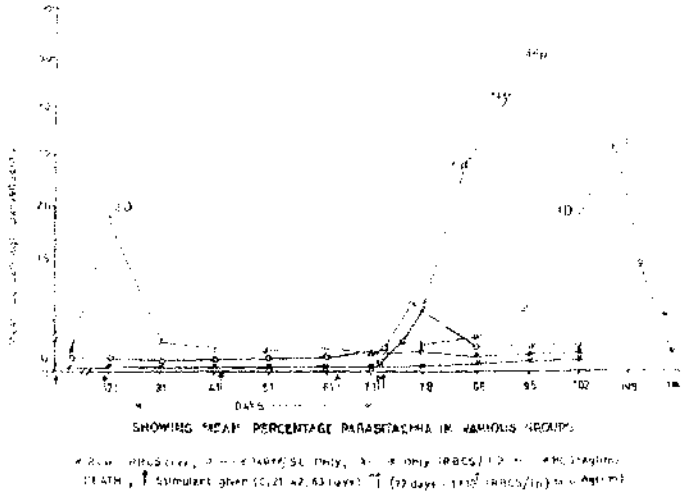


Fig. 3 Experiments on inbred rats showing failure to protection when BCG and parasites are given s.c separately.

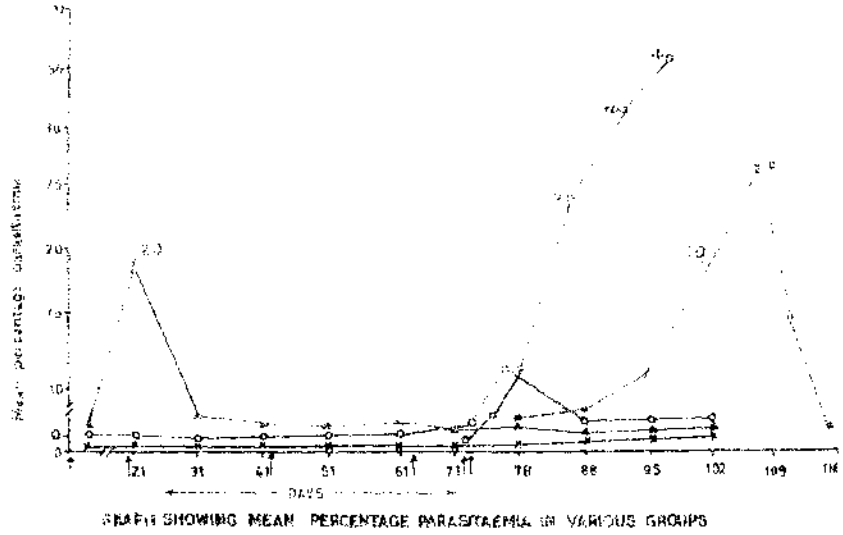


Fig. 4 Illustrating results of different routes of BCG administration in inbred rats.

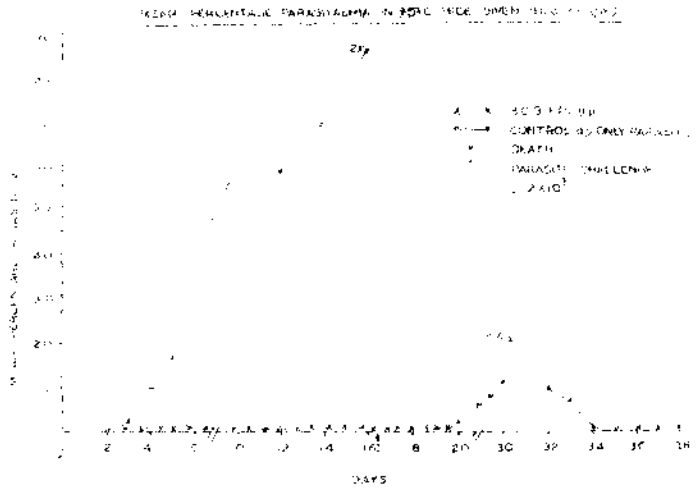


Fig. 4 Protective effect of BCG in BCRC mice known to be non-responsive to BCG.

5. CFA + Parasite + C
6. CFA S. C
7. Parasite given S. C
8. Parasite I.P. (Control)

All experimental animals received infective dose on 49th day i.e., 1 week after three immunizations.

PAGE: Was done in 1% agar (Oxoid Limited) in Tris HCl buffer (0.05 M, pH 8.6) using 10 mV current per slide for 90 minutes.

Preparation of antigen: The antigen was prepared by repeated freezing and thawing of 1×10^8 parasitized cells/ml. Briefly, parasites were collected from 7-10 days infected animals with 50-80% parasitaemia. After separation of leucocytes from the infected blood by dextran treatment, RBCs were washed with normal saline, adjusted to 1×10^8 cells/ml and subjected to 5 cycles of freezing (−70°C) and thawing (37°C). The contents centrifuged at 2000 rpm for 20 minutes and the supernatants were stored at −70°C until use.

Antimalarial antibody (AMA): Either the serum from chronic animals was used, or antimalarial antibody was raised in rabbits as described by Diggs (1966). The rabbits were immunized i.m. twice with 1×10^8 parasitized RBCs in one ml of CFA.

One week after the last immunization their sera tested for antimalarial antibodies by gel diffusion and used for testing presence of antigen in various groups using *CIEP*.

RESULTS

The comparative effect of BCG, *C.parvum* and CFA using repeated doses of adjuvant and parasite at 10 days interval in rats (Fig. 1). BCG given subcutaneously afforded complete protection upto 140 days. Similarly all the animals (6/6) in the CFA group were protected although the parasitaemia reached around 15% after the last parasite challenge. On the other hand, protection observed by *C.parvum* was time bound. The parasitaemia remained low till 90th day after which there was a sharp rise and 3/6 animals died of fulminant infection. The parasitaemia in the control group showed a bimodal peak and all the animals survived till 140 days, i.e. the last day of observation (Fig. 1a).

Even though the immunization schedule was kept constant as in Figure 1, by changing the dose of parasites in immunizing inoculum and final parasite challenge (one week after last immunization) all the

animals survived in this group as well (Table 2). When BCG+parasite (in increasing doses) were given i.v. the animals showed considerable degree of protection as indicated by lowering of parasitaemia. On the contrary, BCG+Ag. (Equivalent to increasing doses of parasites in other groups) when given i.p., the animals did not show protection and at low levels of parasitaemia the animals started dying and similar results were obtained when only Ag. was given as immunizing agent (Fig. 2, 3).

Immunization in mice. Similar experiments were repeated in Swiss inbred ICRC mice. Contrary to the previous reports, we observed a hundred per cent protection when animals were given BCG subcutaneously in 3 doses at 21 days interval followed by a challenge one week after the last immunization (group A).

It was observed that when BCG and parasite were given subcutaneously at different sites, a 66.6% survival could be obtained (Table IV) but when BCG and parasites were mixed and given subcutaneously, a 75% survival was observed. Such a mixture, however, when given intravenously afforded a complete protection against a lethal *P.berghei* infection even after a single immunization. Similarly when CFA mixed with parasites was administered to mice (B_3) it afforded a complete protection.

Thus BCG and Parasites when given *either* subcutaneously or intravenously on 0, 21, 42 days and challenged with a lethal inoculum resisted the infection. However, when BCG protected animals were challenged with a higher dose of parasites (2×10^7/animal i.p.) the parasitaemia went upto to 28% and 2/9 animals died whereas the rest cleared the infection (Figure 4). Thus the protection is linked with the parasite load as well.

In animals immunized with parasites alone, the maximum parasitaemia reached was 12% and 2/10 animals died at peak parasitaemia and the rest also gradually succumbed to infection.

DISCUSSION

The results of the present study indicate that BCG and CFA when given to either inbred rats or mice can protect the recipient from an otherwise lethal *P.berghei* infection and that the protected rats have the capacity to mount adequate antibody response. By changing the immunization schedule and route of immunization it is possible to immunize ICRC mice as well as inbred wistar rats while in the control group all the animals succumbed to infection. Similarly, animals were also protected when CFA was given alone while *C.parvum* afforded only a partial protection (Figs. 1, 2, 3 and Table 1 & 2).

These observations are contradictory to the reports described by Murphy (1981). Many stages of the lying, attenuated, killed or fractionated parasites have been successfully used as antigens. Not only the observed protective immunity is stage and species specific but the effector mechanism in the various host-parasite models is also apparently different. The observed diversities in the effector mechanisms are dependant upon species-specific immunological capability, the type of parasite used (live, killed or fractionated), the state of antigen (particulate, soluble) coupled to the adjuvant and the adjuvant itself (Cohen, 1978).

It has been shown that repeated *P.knowlesi* infection and drug cure leads to acquired immunity associated with chronic relapsing parasitaemia, whereas challenge of rhesus monkeys previously vaccinated with schizont or merozoite antigens in FCA results in complete elimination of parasites (Mitchell et al. 1977). Similarly, mice treated with corynebacterium parvum are shown to be protective against sporozoite challenge (Nussenzweig et al. 1967). However, the basis for this very significant difference between the immune responses induced by vaccination *vis-a-vis* infection has not been clearly elucidated.

In the present study it has been clearly demonstrated that even the ICRC mice treated by a single intravenous dose of BCG and parasites are completely protected.

The non specific immuno-stimulatory effect of BCG is well recognized. It has been reported that a single subcutaneous infection of BCG is not protective against *P.berghei*, whereas intravenous route is

Clark *et al.*, 1976). However, three repeated subcutaneous doses of BCG used in this study were as effective as one i.v. BCG administration used by Clark & Allison (1976). Further, parasite load could be yet another factor modulating the ultimate effect of BCG. It was observed by Bryceson *et al.* (1970) that mice infected with a large number of amastigotes of Leishmania donovani are not protected by BCG. Similarly, Smrkovski *et al.* (1981) reported immuno-supressive effects of BCG against a sporozoite induced *P.berghei* infection. Such a diametrically opposite effect of BCG could result from antigenic competition, blockading of reticulo endothelial system or a massive antigenic load inducing a high zone tolerance (Florentin *et al.*, 1976; Gelford *et al.*, 1976).

Present studies, however, indicate that BCG was the most effective adjuvant even against a high parasite load of 10^7 parasitized erythrocytes. Although, the repeated challenges in an otherwise immunostimulated animals can also act as boosters so that the animals can resist a heavy inoculum, yet the overall protective effect of BCG remains unquestionable. It is superior to the conventional Freund's adjuvant with regard to deleterious local reactions.

REFERENCES

1. Bryceson, A.D.M. (1979): Immunological aspects of clinical leishmaniasis. *Proc.R.Soc.Med.* 63: 1056-1068.
2. Clark, I.A., A.C., Allison, F.E. Cox, (1976): Protection against *Babesia* and *Plasmodium* with BCG. *Lancet* i: 409-310.
3. Cohen, S. (1978). Acquired immunity to the erythrocytic stages of malaria. In Malaria Immunology & Immunopathology Edited by Dan T. Spira & Charles, I. Green balt., p.35-38, published by S. Zarger & Co., Munchen.
4. Diggs, C.L. (1966): Antibodies in malarial infection. *Exp. Parasitol* 16: 209-217.
5. Duker, P., L. Taresay, G. Baschang, (1979): Role of synthetic adjuvants as immunostimulants. *Ann.Rep.Med.Chem.* 14: 146-166.
6. Florentin, I., R.M. Druley, G. Mathe, (1976): Inhibitory effect of BCG on T lymphocyte functions. *Agents and Action* 6: 251-259.
7. Gelford, M., S. Orbach, (1976): Role of BCG in tumor enhancement. *Canc.Immunol.Immunopath.* 1: 39-42.
8. Mitchell, G.H., G.A. Butcher, L. Langherne, S. Cohen, (1977): A freeze dried merozoite vaccine effective against *P.knowlesi* malaria. *Clin.Exp.Immunol.* 28: 276-279.
9. Murphy, J.R. (1981): Host defence in murine malaria. Non-specific resistance to P.berghei generating in response to M.bovis infection or Corynebacterium parvum stimulation. *Infec. Immun.* 33(1): 199-211.
10. Nussenzweig, R. (1967): Increased non-specific resistance to malaria produced by administration of C.parvum. *Exp. Parasitol.* 21: 224-231.
11. Parashar, A., S. Sehgal, R.K. Aikat, S. Naik, (1977). Cell mediated and humoral immunity in P.berghei infection. *Trans.R.Soc.Trop.Med.&Hyg.* 71(6): 474-480.
12. Parashar, A., S. Sehgal, S. Naik, and R.K. Aikat, (1982): Role of macrophage processed antigens in P.berghei model. *Parasite Immunol.* (In press).
13. Smrkovski, L.L. (1981). Effect of route of M.bovis BCG administration on induction of supression of sporozoite immunity in roden malaria. *Infect.Immun.* 31(3): 408-412.
14. Zbar, B., I.D. Bernstein, H.J. Rapp. (1971). Effect of BCG on inducing tumor regression. *Natl.Canc.Inst.* 46: 831-833.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 121-124

Chemotherapeutic Response of *Plasmodium yoelii nigeriensis* to Antimalarial Drugs

ANITA PANDE and S.P. DUTTA

Spontaneous low level of innate resistance of *P. yoelii nigeriensis* to several antimalarials has been observed. In the standard 4 day test, in Swiss mice, the MED of different drugs against *P. yoelii nigeriensis* was found to be as follows: chloroquine 32 mg/kg, mefloquine 4 mg/kg, quinine 150 mg/kg, primaquine 12.5 mg/kg, DADDS 8 mg/kg, pyrimethamine 4 mg/kg, dapsone 2 mg/kg, amodiaquine 16 mg/kg and mepacrine 8 mg/kg. Compared to the sensitivity level of normal strain of *P. berghei*, the strain of *P. yoelii nigeriensis* has shown 4-fold innate resistance to chloroquine and amodiaquine, 2-fold resistance to mefloquine and 1.5 fold resistance to quinine. Since this parasite has innate resistance to several drugs which is not possessed by other rodent parasites, it is suggested that this parasite would serve as a valuable screening model to develop new drugs for activity against multiple resistant strains.

INTRODUCTION

The emergence of multiple drug resistant strains of *P.falciparum* in south east Asia and appearance of some foci of chloroquine resistance in India (Pattanayak *et al.*, 1979; De *et al.*, 1979; Das *et al.* 1979) is causing great concern and thus calls for development of new drugs for the control of drug resistant strains (Puri and Dutta, 1982).

Several rodent malaria parasites such as *P.berghei*, *P.yoelii* and *P.chabaudi* are being currently used for chemotherapeutic screening tests. According to Diggens and Gregory (1969), *P.berghei berghei* NK65, *P.berghei yoelii* 17X and *P.yoelii nigeriensis*, have certain degree of natural chloroquine resistance and thus may serve as useful tool to screen and develop new drugs for the control of chloroquine resistant malaria infections. In the present study, the chemotherapeutic response of *P.y. nigeriensis* to different drugs has been studied.

MATERIAL AND METHODS

The normal strains of *P.yoelii nigeriensis* and *P.berghei* have been maintained by weekly blood passage in Swiss mice. For determining the MED (minimum effective dose) of various antimalarial drugs, different dilutions of drugs were tested in batches of 6 Swiss mice (weighing 20 ± 1 gm). Mice inoculated with 1×10^7 parasitized erythrocytes, were administered the drug orally once a day, from day 0 to +3. On day +4 and +7, the blood smears were prepared and stained with Giemsa. The percent parasitaemia was recorded.

The minimum dose which cleared the blood parasitaemia from day 4 to 7, was considered the MED (Thurston, 1953). All the antimalarials were purchased locally except mefloquine HCl which was obtained from the manufacturers (M/s Roche Products).

RESULTS AND DISCUSSION

The data on the chemotherapeutic response of *P.yoelii nigeriensis* to nine standard antimalarial drugs are presented in Table 1, and the data on response of normal strain of *P.berghei* to these drugs as reported earlier from this institute by Puri *et al.* (1979), Agarwal *et al.* (1979), and Kazim and Dutta (1980) are included in the Table 1 for comparison. Since the strain of *P.berghei* was maintained by weekly blood passage for the last 3 years, the data obtained on the present MED of this strain has been included in Table 1.

The standard 4 day test with chloroquine shows that MED of *P.y.nigeriensis* is 32 mg/kg compared to *P.berghei* (8 mg/kg) and the former is thus four times less sensitive. These results clearly show that *P.y.nigeriensis* has natural chloroquine resistance. On the MED basis, *P.y.nigeriensis* also showed

Accepted for publication 23 December 1982.
Division of Microbiology,
Central Drug Research Institute,
Lucknow.

TABLE 1 Chemotherapeutic response of *P. yoelii nigeriensis* and *P. berghei* (several strains) to different antimalarial drugs

Drug	MED of *P. yoelii nigeriensis*		MED of sensitive strain of *P. berghei* reported earlier (1979-1980)		Present MED of sensitive strain of *P. berghei*	
	Daily dose mg/20g/mouse	Daily dose mg/kg	Daily dose mg/20g/mouse	Daily dose mg/kg	Daily dose mg/20g/mouse	Daily dose mg/kg
1. Chloroquine (base)	0.64	32.0	0.12	8.0[1]	0.16	8.0
2. Mefloquine	0.16	8.0	0.08	4.0[2]	0.08	4.0
3. Quinine	9.0	450.0	5.0	300.0[3]	5.0	300.0
4. Primaquine	0.35	17.5	0.35	17.5[1]	0.35	17.5
5. DADDS	0.16	8.0	0.16	8.0[1]	0.16	8.0
6. Pyrimethamine	0.02	1.0	0.02	1.0[1]	0.02	1.0
7. Dapsone	0.04	2.0	0.08	4.0[2]	0.08	4.0
8. Amodiaquine	0.32	16.0	0.16	8.0[1]	0.08	4.0
9. Mepacrine	0.16	8.0	0.20	10.0[1]	0.16	8.0

MED: Minimum effective dose

[1] = From Puri *et al.* (1979)

[2] = From Kazmi and Dutta (1980)

[3] = From Agarwal *et al.* (1979)

4-fold resistance to amodiaquine which is another 4-aminoquinoline, as compared to *P.berghei*. The sensitivity of *P.y.nigeriensis* to quinine and mefloquine is also lower since their MED has been found to be 450 mg/kg and 8 mg/kg respectively, as compared to the sensitivity of *P.berghei*. The sensitivity of *P.y.nigeriensis* to pyrimethamine, DADDS and primaquine is similar to that of *P.berghei*.

Although the natural resistance of *P.y.nigeriensis* to chloroquine was reported earlier by Diggens and Gregory (1969) and it was further shown to be sensitive to pyrimethamine, the present investigation shows that *P.y.nigeriensis* possesses a natural mode resistant to several antimalarials to which it has never been exposed. Thus, compared to the sensitivity level of *P.berghei*, the *P.y.nigeriensis* exhibits 4-fold resistance to chloroquine and amodiaquine, 2-fold resistance to mefloquine and 1.5 fold to quinine. Further *P.y.nigeriensis* has been found to be more sensitive to dapsone (MED 2.0 mg/kg) compared to *P.berghei* (MED 4.0 mg/kg). Further, it has been found that the drug sensitivity of normal strain of *P.berghei* did not change significantly during the maintenance of the strain in mouse for 3 years by weekly serial blood passage. The present MED values of different drugs against *P.berghei* are the same as reported earlier by Puri *et al.* (1979), Agarwal *et al.* (1979) and Kazim and Dutta (1980). However, the strain has been found to become slightly more sensitive to amodiaquine as shown by decrease of its MED from 8.0 to 4.0 mg/kg.

Several earlier workers have reported the loss of malarial pigment formation in experimentally selected chloroquine resistant strains of *P.berghei* (Peters, 1965; Ladda and Sprinz, 1969 Puri, Agarwal, Kazim and Dutta 1979). However, the present strain of *P.y.nigeriensis* differs from the above strains of *P.berghei*, in the development of normal pigment.

The level of resistance of *P.y.nigeriensis* to chloroquine and other drugs seems to be stable since there has been no change in its drug sensitivity during continuous blood passage over period of one year. The present study suggests that *P.y.nigeriensis* can serve as a valuable screening model to develop new antimalarial drugs against multiple resistant strains.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Nitya Nand, Director, for continued support, and to the Director General, Indian Council of Medical Research, New Delhi for the award of a Fellowship to one of the authors (Mrs. Anita Pande). *P.yoelii nigeriensis* used in the study was received through the courtesy of Prof. P.C.C. Garnham and Mefloquine HCl was received from M/s. Roche Products Ltd., Basel.

REFERENCES

1. Agarwal, S., S.K. Puri and G.P. Dutta, (1979). Selection of pyrimethamine resistant strain of *Plasmodium berghei*. *Ind. J. Med. Res.* 69: 577-582.
2. Das Shilpi, B.G. Roy and S. Pattanayak, (1979). A note on chloroquine resistance tests on *P.falciparum* in Nagaland. *Ind.J.Med. Res.* 70: (Suppl.) 30-33.
3. De, C.M., S.C. Dutta, B. Chowdhury, R.G. Roy and S. Pattanayak, (1979). Results of chloroquine sensitivity tests in *P.falciparum* in some districts of Gujarat, Maharashtra States. *Ind.J.Med. Res.* 70: (Suppl.) 23-26.
4. Diggens, S.M. and K. Gregory (1969). Comparative response of various rodent malarias to chemotherapy. *Trans. Roy. Soc.Trop.Med. & Hyg.* 63: 7.
5. Kazim, M. and G.P. Dutta, (1980). Selection of a sulphanilamide resistant strain of *Plasmodium berghei*. *Ind.J. Med.Res.* 72: 632-636.
6. Ladda, R. and H. Sprinz, (1969). Chloroquine sensitivity and pigment formation in rodent malaria. *Proc.Soc.Exp.Biol. Med.* 130: 524.
7. Pattanayak, S., B.G. Roy, G. Phukan and B.N. Barkakaty, (1979). Chloroquine resistance in *P.falciparum* in Assam State. *Ind.J.Med.Res.* 70: (Suppl.) 14.
8. Peters, W. (1965). Drug resistance in *P.berghei*. Vincke & Lips, 1948. I Chloroquine resistance. *Expt.parasitol.* 16: 80.
9. Peters, W. (1970). Chemotherapy and Drug-resistance (Academic press, London & New York). 876.
10. Puri, S.K., K. Agarwal, M. Kazim, and G.P. Dutta, (1979). Selection of a chloroquine resistant strain of *P.berghei*. *Ind.J.Med.Res.* 70: (suppl.) 85.
11. Puri, S.K. and G.P. Dutta, (1982). Antibiotics in the chemotherapy of malaria. Progress in drug research. Vol. 26: 167-216.
12. Therston, J.P. (1953). The action of antimalarial drugs in mice infected with *Plasmodium berghei*. *Br.J.Pharmacol.* 5: 409.
13. Warhurst, D.C. and R. Killick-Kendrick, (1967). Spontaneous resistance to chloroquine in a strain of rodent malaria (*Plasmodium berghei yoelii*). *Nature*, 213: 1048.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 125-128

Effect of Chloroquine Treatment *in vivo* on the Cyclic AMP Level of Erythrocytes from Normal and *Plasmodium berghei* Infected *Mastomys natalensis*

S. KHARE[1], V.M.L. SRIVASTAVA[1], S. GHATAK[1], S. CHANDRA[2] and A.B. SEN[2]

Cyclic AMP contents of erythrocytes from normal and *Plasmodium berghei* infected (36-42% parasitaemia) *Mastomys natalensis* were determined. Erythrocytes from infected animals registered 2.5 fold increase in cAMP content compared to normal erythrocytes. Treatment of infected animals with chloroquine (5 mg/kg) for 2 days further enhanced cAMP level of erythrocytes. From available evidences it is postulated that when cAMP content of parasitized erythrocytes reach a high critical level, normal metabolic activities are stopped (pleiotypic control by cAMP) leading to death of intraerythrocytic parasites. A sharp decline in cAMP level after 4 days of chloroquine treatment, when no parasitized erythrocytes are found in circulation, strengthens this hypothesis.

INTRODUCTION

Major part of the life cycle of malarial parasite is spent within host erythrocyte, an adaptation not only for being near the source of nutrition, haemoglobin, but also for evading direct host immune reactions. Their habitat within the erythrocytes modifies the host erythrocyte membrane. Besides morphological changes (Aikawa, 1977), abnormalities in the composition of membrane proteins including the disappearance and degradation of certain proteins (Weidekaman *et al.*, 1973) and appearance of some others (Wallach & Conley, 1977) have also been noticed. Recent work from various laboratories has implicated cyclic adenosine monophosphate (cAMP) in the regulation of erythrocyte membrane composition and cellular growth (Konev *et al.*, 1977). cAMP exerts its control by coordinately influencing several biochemical processes e.g. membrane transport and biosynthetic reactions (Posternak, 1974). The present communication reports the results of studies on the level of cAMP in the erythrocytes of normal and *Plasmodium berghei* infected animals and the effect of chloroquine treatment on them.

MATERIAL AND METHODS

Chemicals and reagents: cAMP assay kit was purchased from Radiochemical Centre Amersham, England. Theophylline, propranolol and epinephrine were the products of Sigma Chemical Company, USA while chloroquine was obtained from S.A. Pharmacy, Sagar, India. All other reagents used were of analytical grade.

Animals and infection: The strain of *P.berghei* used in the present study was originally obtained from National Institute of Communicable Diseases, Delhi and since being maintained in Swiss mice and *M.natalensis* by serial syringe passage. The "GRA Giessen" strain *Mastomys natalensis* was originally obtained from Institut fur Parasitologie, Giessen, FDR through the kind courtesy of Late Prof. Dr. G. Lammler[3] in 1974 and since being maintained in CDRI Animal House. Eight week old male *M.natalensis* (35-40g) drawn from the colony of the Institute animal house were infected intraperitoneally (i.p.) with 10^6 parasitized erythrocytes. The animals were housed in plastic cages and received standard pellet diet (Hindustan Lever, Bombay, India) and water *ad libitum*. For assessing the population of infected cells giemsa stained blood smears were used. Drug treated groups of normal and *P.berghei*

Accepted for publication: 15 January 1983.
[1] Division of Biochemistry,
Central Drug Research Institute,
Lucknow.
[2] Division of Parasitology,
Central Drug Research Institute,
Lucknow.

infected animals were obtained by administering chloroquine (5 mg/kg body wt.) intraperitoneally for four consecutive days.

Preparation of RBC suspension: Heparinized blood from 3-6 mastomys per group was pooled and centrifuged at 900 g for 10 minutes at 4°C. Plasma and buffy coat were removed and the cells were washed four times by suspending them each time in 3 vol. of Krebs Ringer bicarbonate solution (KRB) containing 0.1% glucose followed by centrifugation (Hertelendy *et al.*, 1979). Erythrocyte counts were obtained with a haemocytometer.

Production of cAMP: Production is defined as the amount of cAMP in the cells and the medium after 10 minutes of incubation in the presence of 1 mM theophylline.

In order to measure the production of cAMP in erythrocytes a set of three tubes in triplicate was arranged. In tube no. 1 the erythrocyte suspension was incubated with theophylline (10^{-3}M) alone. In tube no. 2 and 3 erythrocyte suspensions were incubated with theophylline + epinephrine (10^{-5}M) and theophylline + epinephrine + propranolol (10^{-6}M) respectively. The final volume of the incubation mixture was made upto 1 ml with KRB (pH 7.4). After 10 minutes incubation at 37°C reaction was terminated by the addition of one volume of cold TCA (12% W/V) with vigorous mixing. Precipitated proteins were discarded by centrifugation at 900 g for 10 minutes the supernate was extracted 3-4 times with water saturated diethyl ether to remove TCA and lyophilized to dryness in Leybold Heraeus freeze drier. The contents were reconstituted in cAMP assay buffer (Tris-EDTA buffer, 0.05 M, pH 7.5 containing 4 mM EDTA) in a manner so that 50 µl samples contained cAMP in the range of 1-16 p mol. A calibration curve was drawn using standard cAMP supplied with the assay kit (Amersham, U.K.) in the range of 1 to 16 p mol. With the help of this calibration curve cAMP contents of the above reconstituted samples were determined.

Assay of cAMP: The instructions supplied with the assay kit were strictly followed for the measurement of cAMP essentially according to the techniques of Gilman, 1970 and Brown *et al.*, 1971.

RESULTS

Tables 1 & 2 present the results of studies on the level of cAMP in erythrocytes from normal and infected animals and the effect of chloroquine treatment thereon. The levels of cAMP in erythrocytes have been estimated in the presence of theophylline, epinephrine and propranolol. Theophylline was included in the incubation media for these studies to inhibit the degradation of cAMP by enzyme phosphodiesterase. Epinephrine enhanced the cAMP production, an action mediated through β-adrenergic receptors while propranolol, a β-blocker, blocked the activation caused by epinephrine. Levels of cAMP are expressed in table as pmoles/10^8 erythrocytes/10 minutes.

As would be evident from the Table 1 & 2 in the presence of theophylline (10^{-3}M) alone the cAMP contents of erythrocytes of normal and infected animals were 20 and 50 pmoles respectively. Presence of epinephrine (10^{-5}M) enhanced the level to 37 and 102 pmoles. Propranolol (10^{-6}M) exhibited an effect by nullifying the stimulation caused by epinephrine and the levels came down to 21 and 53 pmoles respectively.

Two days of treatment with chloroquine caused a mild increase in cAMP contents of erythrocytes of normal animals whereas the increase in the erythrocytes of infected animals was marked. Cyclic AMP levels of 29 and 107 pmoles were recorded in the presence of theophylline alone. Epinephrine increased the production of cAMP of erythrocytes from normal and infected animals to 53 and 234 pmoles respectively whereas in the presence of propranolol the levels came down to 31 and 162 pmoles.

Four days of treatment with chloroquine further enhanced the cAMP content of erythrocytes of normal animals and a level of 32 pmoles was registered in presence of theophylline alone. With epinephrine and propranolol levels of 57 and 34 pmoles were recorded respectively. In infected group of animals, levels of 63, 102 and 66 pmoles were found in the presence of theophylline, epinephrine and propra-

Table 1. Effect of Chloroquine Treatment on cAMP Level of Erythrocytes from Normal *M. natalensis*.

Additions	cAMP (pmoles/10⁸ erythrocytes/30 minutes)		
	No Treatment	2 Days of drug treatment	4 Days of drug treatment
Theophylline (10^{-3}M)	20.1 ± 1.5	20.9 ± 0.9	22.3 ± 0.86
Theophylline + Epinephrine (10^{-5}M)	36.7 ± 1.1	53.2 ± 2.2	56.7 ± 1.1
Theophylline + Epinephrine + Propranolol (10^{-6}M)	20.7 ± 2.1	30.7 ± 1.0	34.0 ± 0.5

Results represent mean ± SE of four determinations.

Table 2. Effect of Chloroquine Treatment on cAMP Level of Erythrocytes from *P. berghei* infected *M. natalensis*.

Additions	cAMP (pmoles/10⁸ erythrocytes/30 minutes)		
	No treatment*	2 Days of drug treatment**	4 Days of drug treatment***
Theophylline (10^{-3}M)	50.0 ± 2.7	106.9 ± 1.2	63.2 ± 1.9
Theophylline + Epinephrine (10^{-5}M)	102.0 ± 2.1	234.0 ± 7.1	102.4 ± 3.8
Theophylline + Epinephrine + Propranolol (10^{-6}M)	53.1 ± 1.2	167.0 ± 2.0	66.3 ± 6.5

* Erythrocytes obtained from animals showing 38-42% parasitaemia.

** Erythrocytes obtained after 2 days of drug treatment from animals showing 10-15%.

*** Erythrocytes obtained after 4 days of drug treatment from animals showing no parasitaemia.

Results represent mean ± SE of four determinations.

nolol respectively. It is pertinent to mention here that after 2 days of chloroquine treatment infected animals recorded 10-15% parasitaemia while after 4 days of drug treatment animals became parasite free.

DISCUSSION

Recent reports from various laboratories have shown that when cAMP is present at higher concentration cells assume a quiescent state characterized by low levels of active transport of nutrients, rapid fatty acid and protein degradation and low levels of RNA and protein synthesis (Kram *et al.*, 1973; Kram and Tomkins, 1973). In general high cAMP concentration inhibits growth. Results obtained in the present study indicate that chloroquine treatment significantly increases the cAMP contents of erythrocytes of infected group of animals (2 days post treatment). Reports regarding action of chloroquine show that drug treatment inhibits the transport of nutrients across the erythrocytes (Sherman and Tanigoshi, 1972), impairs the RNA and protein synthesis (Warhurst and Williamson, 1970; Richards and Williams, 1975) which in turn causes death of the intracellular parasite. After chloroquine treatment increased cAMP contents of erythrocytes of infected animals might be responsible for these disturbed metabolic activities. Probably drug treatment increases the cAMP contents of erythrocytes of infected animals to such a critical level (as observed in the present study) that normal metabolic activities are disturbed, thereby producing an environment which is not suitable for the growth and development of the parasite, causing damage and ultimately leading to their death. Although infection itself causes slight increase in cAMP contents of erythrocytes (self defence mechanism operating in the host), the level may not be high enough for restricting the normal metabolic activities. However, as soon as chloroquine is administered, it increases the cAMP production significantly. After 4 days of chloroquine treatment few or no infected cells could be observed in circulation which accounts for marked low levels of cAMP in the erythrocytes. It is pertinent to mention here that the presence of cAMP in the parasite (*P.berghei*) could not be detected by the procedure employed hence observed levels in the erythrocytes were independent of parasitic contribution.

It may, therefore, be inferred that chloroquine treatment significantly increases the intraerythrocytic concentration of cAMP which in turn checks the growth and development of the parasites.

REFERENCES

1. Aikawa, M. (1977). Variations in structures and function during the life cycle of malarial parasite. *Bull.WHO.*, **55**: 139-156.
2. Brown, B.L., J.D.M. Albano., R.P. Ekins and A.M. Scherzi (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3', 5'-cyclic monophosphate. *Biochem. J.* **121**: 561-562.
3. Gilman, A.G.A. (1970). Protein binding assay for adenosine 3', 5'-cyclic monophosphate. *Proc. Natl.Acad.Sci.* **67**: 305-312.
4. Hertelendy, F., M. Toth and C.D. Fitch (1979). Malaria enhances cyclic AMP production by immature erythrocytes *in vitro*. *Life Sci.*, **25(5)**: 451-455.
5. Konev, S.V., I.D. Volotovaskii., V.S. Finin., A.V. Kulikov., V.A. Kirillov and E.I. Zaichkin (1977). The structural transition of erythrocyte membranes induced by cyclic AMP. *Biochim. Biophys.Acta.* **470**: 230-241.
6. Kram, R. and G.M. Tomkins (1973). Pleiotypic control by cyclic AMP: Interaction with cyclic GMP and possible role of microtubules. *Proc.Natl.Acad.Sci.* **70(6)**: 1659-1663.
7. Kram, R., P. Mamont and G.M. Tomkins (1973). Pleiotypic control by adenosine 3', 5'-cyclic monophosphate: A model for growth control in animal cells. *Proc.Natl.Acad.Sci.* **70(5)**: 1432-1436.
8. Posterank, T. (1974). Cyclic AMP and cyclic GMP. *Ann.Rev. Pharmacol.* **14**: 23-34.
9. Richards, W.H.G. and S.G. Williams (1975). Malaria studies *in vitro* III: The protein synthesising activity of *Plasmodium falciparum in vitro* after drug treatment *in vivo*. *Ann. Trop.Med.Parasit.* **69(2)**: 135-140.
10. Sherman, I.W. and T. Tanigoshi (1972). Incorporation of C^{14} amino acids by malaria (*Plasmodium lophurae*) V. Influence of antimalarials on the transport and incorporation of amino acids. *Proc. Helminthol.Soc. Wash.* **39**: 250-260.
11. Wallach, D.F.H. and M. Conley (1977). Altered membrane proteins of monkey erythrocytes infected with simian malaria. *J.Mol.Med.* **2**: 119-136.
12. Warhurst, D.C. and J. Williamson (1970). Ribonucleic acid from *P.knowlesi* before and after chloroquine treatment. *Chem.Biol.Interactions* **2(2)**: 89-106.
13. Weidekaman, E., D.F.H. Wallach, P.S. Linn and J. Hendricks (1973). Erythrocyte membrane alterations due to infection with *Plasmodium berghei*. *Biochim.Biophys.Acta* **323**: 539-546.

Indian Journal of Malariology
Vol. 19, Dec 1982, pp. 129-134

Detection and Incrimination of *Anopheles minimus* Theobald 1901 as Malaria Vector in the Foothill Areas of Nagaland, India

V.N. BHATNAGAR[1], S.R. DWIVEDI[1], B.G. MISRA[2] and M. DAS[1]

Anopheles minimus, the chief malaria vector in the foot hills extending from U.P. to the North Eastern States before the national Malaria Control, Eradication Programmes were launched, was detected in the foot hill areas of Nagaland in human dwellings during the course of investigations carried out in 1982 in Dimapur (Nagaland). Dissections showed sporozoites and oocysts in the species.

INTRODUCTION

Anopheles minimus had been found to be the most dangerous and chief vector of malaria in the foot hills of Himalayas extending from U.P. to Assam, because of its predominantly domestic habits and preference for human blood. (Ramsay *et al.*, 1936, Iyengar 1940, Viswanathan *et al*, 1941, Covel 1944, Misra and Dhar 1955 and Misra 1956).

However later studies, though limited, showed the absence of the species from these areas and was therefore presumed to have almost disappeared. Various factors viz. extensive cultivation and deforestation were attributed to its disappearance in U.P. Terai (Chakrabarti and Singh 1957).

Studies carried out in Assam and Meghalaya also showed the absence of the species from the area. (NICD annual report 1971, Rajagopal 1976) Almost total disappearance of *A.minimus* from these areas was attributed to extensive and repeated rounds of DDT spray under National Malaria Control/Eradication Programme commencing from 1953, coupled with extremely high susceptibility of the species to the insecticide.

Studies undertaken on the possible role of other vectors, though limited in scope, had shown *A.philippinensis* and *A. balabacensis* as vectors in Meghalaya, and Arunachal Pradesh. (NICD annual report 1971, Rajagopal 1976, Sen *et al.*, 1973).

However, in view of the persistence of malaria in the North Eastern Region, systematic observations were started under the aegis of *Plasmodium* falciparum Containment Programme (assisted by WHO/SIDA) to study the role of different anopheline vectors in the area. The results of these observations are presented in this paper.

MATERIAL AND METHODS

Two villages, viz. Chumukedima and Tenyiphe situated in the foot hills about 22 kilometres in the east of Dimapur towards Kohima, were selected for the studies. The population of these villages comprises of Naga tribes and Nepalese settlers. The area had been under DDT spray since 1958, but there was no spray in the index villages since 1981 because of the studies.

Day time catches were made fortnightly from fixed and random catching stations between 6 AM to 11 AM, spending 30 minutes in each, by means of an aspirator and a flash light. Night resting catches were also made fortnightly from dusk to dawn in human dwellings and cattle sheds, by collecting for 15 minutes in every hour.

Biting activity was observed by making the human bait sleep in a mosquito net, with one side left partially open to facilitate entry of the mosquitoes.

Accepted for publication, 24 January 1983.

[1] National Malaria Eradication Programme,
22-Sham Nath Marg,
Delhi-110054.

[2] PfCP, Zone-I, Pasteur Hill,
Shillong, (Meghalaya).

Table 1.—Indoor resting catches of *Anopheles minimus*. Village—Tenyphe IV.

MONTH	NIGHT CATCHES								
	Human dwellings			Cattle shed			Human dwelling		
	Time spent hours	Total collected	MHD	Time spent hours	Total collected	MHD	Time spent hours	Total collected	
JANUARY '82	6	NIL	-	6	1	0.16	8	NIL	
FEBRUARY '82	6	NIL	-	6	2	0.33	8	NIL	
MARCH '82	6	NIL		6	NIL		-	-	
APRIL '82	5	22	4.4	6	1	0.16	10 hrs. 30 min.	53	
MAY 82		-			-		8	43	
JUNE '82	10 hrs. 30 min.	69	3.3	6	1	0.16	8	11	

NIL = No specimen of *A. minimus* was encountered, even though other species were captured.

(-) = Not done

* = Collected from a house which had a malaria positive case.

(#) = All the specimens collected were not dissected because 1) most of them were fresh specimens and 2) on account of logistic difficulties.

Table 2.—Indoor biting activity of *Anopheles minimus*. Dusk to dawn observations

Time	6—7pm			7—8pm			8—9pm			9—10pm			10—11pm		
Number Collected in April	UF	F	T	UF	F	T	UF	F	T	UF	F	T	UF	F	T
	Nil	Nil	Nil	2	1	3	2	4	6	4	2* 1 (G)	7	2	Nil 1 (HG)	3
June	Nil	Nil	Nil	3	Nil	3	3	5* 1 (G)	9	nil	Nil	Nil	2	Nil+ 2 (HG)	4

(—) = Not done

= No catches made after 1 am.

IRC Dimapur (Nagaland) (1982).

DAY CATCHES

	Cattle Shed			Dissections			
MHD	Time spent hours	Total collected	MHD	Total dissected	Gland	Gut	REMARKS
	4	NIL	—	—			
	4	NIL	—	—			
5.05	4	NIL	—	5(*)	Neg.	Neg	
7.1	4	NIL	—	7(*)	Neg.	Neg.	*
1.3	4	NIL		12(*)	1	1	*

during April-June 1982. Village Tenyophe, IRC-Dimapur (Nagaland).

11 - 12pm			12 to 1am			1- 2am			2 3am			3- 4am			4--5am		
UF	F	T	UF	F	T	UF	F	T	UF	F	T	UF	F	T	UF	F	T
4	1- 3(HG)	8	2	5- 2(HG)	9	-	--	(*)			-					--	--
Nil	Nil	Nil	1	2	3	Nil	5+ 1(HG)	6	Nil	7+ 2(HG)	9	Nil	3	3	Nil	Nil	2 (G)

Mosquitoes found resting inside the net were collected in every hour for about 15 minutes.

Blood fed females were exposed to 4% DDT impregnated paper utilising WHO test kit for determining susceptibility of adult mosquitoes to this insecticides.

RESULTS

Indoor resting catches of *A.minimus* in the investigation area is put up in Table 1

During January and February no specimen of *A.minimus* was detected in human dwellings during the day time collections but could be collected from human dwellings in the day time catches during April and May with per man hour density of 5.05 and 7.1 respectively. The specimens captured were mostly of the younger age group. It was also found that most specimens of *A.minimus* were encountered in a particular hut used by labourers was for sleeping at night, during the day the hut was uninhabited. The species was found resting on walls near the bamboo cots and underneath the cots. The breeding of this species was detected in slow moving stream near the village.

During June, as the monsoon set in full swing the stream was flooded, which resulted in decrease in the adult density, which went down to 1.3 per man hour. However the specimens captured were mostly older ones and of the 12 specimens dissected one specimen was found positive for sporozoites and oocysts thereby giving an overall sporozoite rate of 8.3% per cent during the month.

In the night collection no specimen of *A.minimus* or any other species could be detected from human dwellings from January to March because, to keep the huts warm, cow-dung cake fire was kept burning throughout the night. From April onwards *A.minimus* density in human dwellings showed an appreciable increase in the night, ranging between 4.7 to 6.5 per man hour.

In cattle sheds a few unfed specimens could be captured in early part of the night during the observation period.

The data of indoor biting activity of *A.minimus* are given in Table-2. It was observed that *A.minimus* entered the houses often at 7 p.m. (after about 2 hours of dusk). Two peaks in biting were detected, one between 8 to 10 p.m. and the other between 1 to 4 a.m. In addition some half gravid and gravid specimens were also captured from inside the net.

Results of Susceptibility tests to DDT , showed the species to be highly susceptible to DDT as all the specimens died during the exposure period. There was no mortality in the control.

DISCUSSION AND CONCLUSION

These observations would indicate that *A.minimus*, which seemed to have disappeared from the North eastern regions, had reappeared and possibly re-established itself in the region, particularly in areas with poor spray coverage. Recently the species has been found in foot hills in Goholkona and Sonapur areas of Assam. (Karim 1981 and Matin 1982, personal communication)

These results also show that the species is still highly domestic, as it rests and feeds indoors, and is highly susceptible to DDT. A thorough insecticidal coverage sustained for a period of 3 years, should help in bringing down the disease incidence considerably. However, more intensive investigations are needed in the area to work out the bionomics of the species as well as the role of other important vector species viz. *A. halabacensis*, *A. philippinensis*, *A. annularis*, *A. culicifacies*, *A. maculatus* which had already been established as vectors in the region (Viswanathan *et al.* 1941, Sen *et al.* 1973 & Rajagopal 1976).

ACKNOWLEDGEMENT

The authors are thankful to the Director of Health Services Nagaland, Dr. R. Shaiza, Assistant Director (Malaria), Nagaland and Dr. P.D. Gogoi, Zonal Malaria Officer, Dimapur for according facilities of accommodation etc., and the staff members of the Entomology Research Cell, PfCP, Dimapur for their assistance in collection of data.

REFERENCES

1. Anonymous (1971). 'Investigation on the behaviour pattern of Anopheles Vectors and Epidemiology of malaria in Burnihat, Meghalaya.' N.I.C.D., Annual Report.
2. Chakraborty, A.K. and N.N. Singh, (1957) 'The probable causes of disappearance of *A minimus* from Terai area of Nainital district of Uttar Pradesh.' *Bull.Nat.Soc.Ind.Mal. Mos.Dis.* 5: 32-85.
3. Covell, G. (1944). 'Notes on distribution, breeding places, adult habits and relation to malaria of the Anopheline mosquitoes of India and Far East.' *J.Mal Inst.Ind.*, 5: 399-434.
4. Iyengar, M.O.T. (1940). 'Further observations on vectors of Malaria in Bengal.' *J.Mal.Inst.Ind.*, 3: 115-123.
5. Misra, B.G. (1956). 'Malaria in North Eastern Frontier Agency' *Ind.Jour.Mal.* 10: 331-347.
6. Misra, B.G. and S.E. Dhar (1955). 'Malaria in Tripura State.' *Ind.Jour.Mal.* 9: 111-123.
7. Ramsay, G.C., S.N. Chandra, and B.A. Lamprell (1936) 'A record of an investigation to determine the androphilic indices of certain *Anopheles* mosquitoes collected in Tea Estate in Assam and N. Bengal.' *Rec.Mal.Surv.Ind.* 6: 49-52.
8. Rajagopal, R. (1976) 'Studies on persistent transmission of malaria in Burnihat, Meghalaya.' *J.Com.Dis.* 8: 235-245.
9. Rao, T.R. (1981). *The Anophelines of India* pp. 32, 343-348 (W.O. Judge Press, Bangalore).
10. Sen A.K., V.M. John, K.S. Krishnan, and R. Rajagopal (1973) 'Studies on malaria transmission in Firap district, Arunachal Pradesh.' (NEFA) *J.Com.Dis.* 2: 90-110.
11. Sharma M.I.D., (1958). Laboratory and field development of resistance in mosquitoes. *Ind.J.Mal.* 12: 401-411.
12. Viswanathan, D.K., S. Das., and A.V. Ommen (1941). 'Malaria carrying anophelines in Assam.' *J.Mal.Inst.* 4: 297-306.

SHORT NOTES

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 137-138

Modified Plan of Operation and Its Impact on Malaria

R. C. PRETY, V.K. GUPTA and V.P. SHARMA

In April 1977, Government of India implemented the modified plan of operation (MPO) for the control of malaria (Pattanayak and Roy, 1980). As per the guidelines of MPO, operational units comprising of 1 million population and classical phasing of malaria eradication (i.e. preparatory, attack, consolidation and maintenance phases) were abolished. Malaria control was decentralised and district boundaries became the administrative units. Former vertical line of administrative and technical control was changed to a combination of vertical and horizontal pattern i.e. at the State and Zonal level, unipurpose officers and at the district and Primary Health Centre level, multipurpose officers were placed. They were supported by unipurpose workers like District Malaria Officer, Assistant Malaria Officer and Malaria Inspector. There was a multipurpose worker at the periphery who was responsible for the implementation of all the National Health Programmes including malaria control for 8,000 to 10,000 population. The centralised laboratory services were decentralised to the Primary Health Centres (PHCs) with additional responsibilities of providing laboratory facilities for other national health programmes and clinical laboratory support to the medical officers in PHCs. This was to be implemented in phases and it was envisaged that by 1982 the entire country will be covered under this scheme. It was also envisaged that areas with 2 or more annual parasite incidence (API) would be sprayed with suitable residual insecticides and areas with <2 API would not be sprayed except for the focal spraying. MPO was implemented with the following objectives.

(1) prevention of death due to malaria;
(2) reduction in morbidity due to malaria;
(3) maintenance of the industrial and green revolution; and
(4) retention of the achievements gained.

MPO envisaged organizing a drug distribution system so that anti-malarials were available to every one on the doorsteps. Distribution of the drug was organized through the Fever Treatment Depots (FTDs), Drug Distribution Centres (DDCs), voluntary agencies and the passive institutions. In 1981 a study was undertaken to see the impact of the modified plan of operation on malaria. Results of this study are reported below.

Spleen surveys were conducted during September and October in Banthla and Makanpur villages under Loni PHC (District Ghaziabad, Uttar Pradesh, where MPO had been in force since 1977 and multipurpose worker (MPW) scheme since 1978). Afebrile children of 2-9 year age group (from schools and villages) were examined for the spleen enlargement by the Hackett's method (Christophers *et al.* 1958). Out of a total of 517 children examined, 152 had enlarged spleen (29.4 per cent) with an average enlarged spleen of 2. Blood smears of these children were stained with JSB and examined for the presence of malaria parasite. Examination of 100 and 200 fields in thick and thin smears respectively revealed that 35 blood smears were positive

Accepted for publication 13 July 1982.
Malaria Research Centre (ICMR),
22 Sham Nath Marg,
Delhi-110054

for the malaria parasite (slide positivity rate was 6.8 per cent), and of these 16 were positive for *P.falciparum* (i.e. Pf percentage was 45.7).

Fever surveys were carried out in November in 5 villages under three PHCs of Ghaziabad district covering one village in a day. House to house visits were made to collect blood smears from febrile cases. About 53 per cent population (6,200 out of 11,725) was covered during the survey. It was revealed that blood smears from fever cases were being missed by the MPW in most of the houses. A total of 519 blood smears were collected, stained and examined as described above. There were 236 blood smears positive for malaria parasite (i.e. slide positivity rate was 45.5 per cent), and of these 227 smears were positive for *P. falciparum* (i.e. Pf percentage was 96.2). History of the fever was recorded and it was revealed that the duration of morbidity in malaria positive cases generally extended up to 23 days before spontaneous remission occurred.

Study of the functioning of drug distribution system showed that out of a total of 765 FTDs and 674 DDCs established in Ghaziabad district, only 39 FTDs and 55 DDCs were functioning during October. FTDs which were required to prepare blood smear for microscopic examination before giving drug were not doing so (i.e. district data showed that during 1980, out of 58,988 fever cases recorded by FTDs only 6,795 blood smears were made). The F.T Ds were functioning as DDCs.

Through questionnaires it was revealed that in Ghaziabad district, among the FTD and DDC holders, timings of work were not fixed of 63 per cent holders, 35 per cent holders had not accepted this service willingly and 87 per cent holders did not receive regular supplies of the antimalarials and microslides, etc. Among the private practitioners in Ghaziabad district, Delhi and Faridabad district (Haryana state), it was revealed that of the 126 practitioners, 105 (83.3 per cent) were not using standard 4-aminoquinoline regime, 41 (32.5 per cent) were using new drugs like Metakelfin (Sulphamethopyrazine+Pyrimethamine), 110 (87.3 per cent) were not getting the blood smears examined, and 107 (85 per cent) were not insisting on the radical treatment.

The study pointed out that at least in the study areas (i) MPO was not functioning satisfactorily (ii) MPW was missing fever cases (iii) presence of asymptomatic carriers (iv) Long duration of morbidity was associated with *P. falciparum* infections. This may be the result of either high failure rate or inadequate intake of chloroquine (v) need of educating the private practitioners and (vi) that similar studies were indicated in other regions.

REFERENCES

1. Christophers, S.R., J.A. Sinton and G. Covell, (1938). How to do a Malaria Survey. *Hlth. Bull.* No. 14, Malaria Bureau, No. 6 (Govt. of India Press).

2. Pattanayak, S. and R.G. Roy, (1980). Malaria in India and the Modified plan of operation for its control. *J.Com.Dis.* **12(1)**: 1-14.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 139-142

Preliminary Studies on Irrigation Malaria

V.P. SHARMA[1] and H.C. UPRETY[1]

In India a network of dams, canals and their distributaries was established for increasing acreage under irrigation. The gross area under irrigation in 1960-61 was 29.05 m ha. This was increased to 37.10 m ha in 1968-69, 40.20 m ha in 1973-74, 52.07 m ha in 1977-78 and 57.02 m ha in 1976-80. Early work on irrigation malaria had shown that irrigation increases the incidence of malaria (Gill 1930, Russell *et al.* 1938). The impact of this spectacular increase in irrigation on the receptivity and resurgence of malaria was suggested by Sharma and Mehrotra, (1982 a,b). A study was therefore initiated to compare the prevalence of malaria in villages with and without canals. Results of this study are reported below.

Study sites selected were in district Meerut (UP) and Gurgaon (Haryana). People of these villages were of comparable socio-economic status and similar cultural habits. In Meerut villages there was a network of canals and study villages were situated within 1-2 kms of the canal irrigation. In Gurgaon villages, there was no canal irrigation upto at least 40 kms in any direction. *A.culicifacies* was the vector species of these villages which breeds in stagnant pools, irrigation waters, puddles, borrowpits and rainwater collections. In 1981-82 a large number of specimens of *A.culicifacies* were incriminated from Haryana and U.P. villages (D.S. Choudhury, Personal communication). The vector has become resistant to DDT and HCH so that spraying of these insecticides is not fully effective. To study the prevalence of malaria fever surveys were carried out in June and October 1982 in the same population and house to house visits were made to collect blood smears. Survey in each district lasted for 8-10 days. Fever cases were given presumptive treatment of four chloroquine tablets (proportionate dose to children). Blood smears were fixed and stained with JSB and examined under the microscope for the presence of malaria parasites. Results of this study are given in Table 1.

Study revealed (Table 1) that villages with canal irrigation had higher fever rate i.e., 3.16% against 1.44% in villages without canals. The incidence of malaria was also high in the canal irrigated villages i.e., 57 cases against 10 in June (5.7 × more) and 81 cases against 9 in October (9 × more). It was also revealed that in October, the incidence of falciparum malaria was extremely high in canal irrigated villages i.e., 50 cases against 3. It was therefore clear that villages with canals were more prone to malaria.

Rao (1945) and Rao and Nassiruddin (1945) reported that in villages under Mandya district (Mysore) the average spleen rate rose from 15.3 per cent to 50 to 90 per cent with the opening of Visvesvaraya canal. There was a twenty fold increase in deaths due to fever and within three years of the introduction of irrigation the conditions had settled down to a state of hyper-endemicity.

Accepted for publication: 1 December 1982.
[1] Malaria Research Centre (ICMR),
22 Sham Nath Marg,
Delhi-110054.

Table 1 Prevalence of malaria in Canal and non-canal irrigated villages.

Month (1982)	Population contacted	No. of wells	No. of tubewells	Blood smears collected from febrile cases	Total malaria parasite positive cases	*P. vivax*	Blood smears positive for *P. falciparum*	Slide positivity rate
1. Villages with canal irrigation* (canals within 1-2 kms)								
June	10020	37	141	306	57	56	1	18.62
Oct.	10048	37	141	318	81	31	50	25.47
2. Villages without canal irrigation** (canals beyond 40-kms)								
June	10058	65	914	155	10	10	0	6.45
Oct.	10249	65	914	154	9	6	3	5.84

Villages: * Rataul, Dagarpur, Fakharpur & Mavikalen in Bagpat Tehsil, Distt. Meerut, U.P.

** Babra Bakipur, Abajhund Sarai, Khawaspur, Jamalpur, Taj Nagar, Fazilpur Badli, Joniawas and Jundola in Tehsil and Distt. Gurgaon, Haryana.

Note: Gurgaon villages were small compared to Meerut villages. Counting of wells and tubewells was done in about equal area in both the category of villages.

Early work on irrigation malaria in Punjab (Gill 1930) and Pattukkottai (Russell *et al.* 1938 and Russell 1938) had shown that there was intimate association of *A.culicifacies* and irrigation water, and that irrigation increases the incidence of malaria. Russell (1938) described some specific ways in which irrigation brings malaria and observed that it was not irrigation *per se* but defective and untidy irrigation, which by misplacing to the advantage of certain anopheline mosquitoes, generates malaria. Waste water was an important source of malaria. In the present study specific reasons for the high prevalence rate of malaria in villages with canal irrigation were not investigated. It was observed that irrigation needs in villages without canals were being met by wells/tubewells, the number of which was considerably high (see Table 1). It was therefore likely that defective and untidy irrigation was the cause of high prevalence of malaria in canal irrigated villages. Further studies are indicated.

REFERENCES

1. Gill, C.A. (1930) The relationship of canal irrigation and malaria. *Records Malar. Surv. India* **1(3)**: 417-422.
2. Rao, B.A. (1945) Malaria in the Irwin canal area, Mysore state Part I: 101-108. *Jour. Mal. Inst. Ind.* **6**: pp. 101-108.
3. Rao, B.A. and M. Nassiruddin, (1945). Malaria in the Irwin canal area, Mysore state Part II. *Jour. Mal. Instt. Ind.* pp. 109-128.
4. Report of the working group on minor irrigation for formulation of the VI Plan proposals for the year 1980-85. Minor Irrigation Division. Ministry of Agriculture, p. 7 (Government of India, 1980).
5. Russell, P.F. (1938). Malaria due to defective and untidy irrigation : A preliminary discussion. *Jour Malar. Inst. India.* **1(4)**: 339, 349
6. Russell, P.F., M.K. Menon, T. Ramachandra Rao (1938). Epidemiology of malaria in Pattukkottai Taluk, Tanjore District, Madras Presidency, India. *Jour. Malar. Inst. India.* **1(3)**: 285-326.
7. Sharma, V.P. and K.N. Mehrotra (1982a). Return of Malaria. *Nature (Lond.).* **216**: 210.
8. Sharma, V.P. and K.N. Mehrotra (1982b) Malaria Resurgence *Nature (Lond.).* **300**: 212.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 143-144

Staining of Sporozoites from Infected Mosquitoes

D.S. CHOUDHURY[1] and S.K. GHOSH[1]

At the Malaria Research Centre, attempts are being made to incriminate and re-incriminate vectors of malaria in different parts of the country. During the last two and half years, 133 natural infections of sporozoites were detected in the salivary glands of *Anopheles culicifacies* from Haryana, Delhi and Uttar Pradesh (Ghaziabad and Terai region). Sporozoites have also been found in the salivary glands of two *A.fluvitalis* collected from U.P. Terai. Some of the slides containing these sporozoites were fixed with methyl alcohol and stained by J.S.B. technique (Jaswant Singh and David 1949, Jaswant Singh and Puri 1958). It was observed that considerable number of the sporozoites were lost during staining. A modified staining technique was therefore developed to obtain uniformly good results without the loss of sporozoites. This technique is described below.

The formal saline solution used in the study consisted of one part of formalin (40 per cent formaldehyde) and 9 parts of normal saline. Slide containing the glands with sporozoites was fixed by running a few drops of formal saline solution under the coverslip. After the formal saline had completely dried, the coverslip was removed with the help of two dissecting needles and area containing the glands was marked by a glass marking pencil. The formal saline was then washed thoroughly by at least three changes of distilled water. For staining, three drops of the stock solution of Leishman stain were added on the marked area of the slide and on the coverslip containing parts of the glands. It was allowed to act for one minute. Subsequently, the stain was diluted by adding seven drops of distilled water. The diluted stain was allowed to act for 30 minutes. During this period, the stain was slowly agitated with the help of a pasteur pipette to avoid scum formation. The slide was thoroughly washed with repeated changes of distilled water till all the stain deposits had been completely removed. When dried, permanent mounting was done using the D.P.X. mounting fluid. Uniformly good results were obtained by using this technique (Fig. 1) and the loss of the sporozoites was negligible.

Accepted for publication: 1 December 1982.

[1] Malaria Research Centre (ICMR),
22 Sham Nath Marg,
Delhi-110054.

Fig. 1 - Showing sporozoites coming out of the salivary glands of mosquito. (× 1,400).

REFERENCES

1. Singh, J. and David, (1949). Staining and restaining of oocysts and sporozoites from infected mosquitoes. *Ind. J. Mal.* 3(4): 349-352.

2. Singh, J. and I.M. Puri, (1958). How to do a malaria survey. Health Bulletin No. 14. Malaria Bureau No. 6. 6th Edition. 43-44.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 145-146

Susceptibility of *Anopheles philippinensis* to DDT and Dieldrin in Assam

J. DUTTA CHOUDHURY[1] and P.R. MALHOTRA[2]

Susceptibility of a vector to insecticides is an essential prerequisite in a vector control programme. Susceptibility of *Anopheles philippinensis* Ludlow, an incriminated malaria vector, to DDT has been recorded recently from north-eastern region of India (Nawab Singh and Chakraborty, 1979, Sundaram *et al.* 1980). In this communication, results of susceptibility tests with adults *A.philippinensis* against diagnostic dosages of DDT and dieldrin in Assam, are presented. The studies were carried out in different months from 1977 to 1980 in various localities under 12 Primary Health Centres in Cachar, North-Cachar Hills and Karbi-Anglong districts of Assam.

Regular spraying of DDT had been carried out since 1958 as per NMEP schedule. Besides DDT, HCH had also been carried out in late sixties and early seventies. Modified Plan of Operation was implemented in Assam from 1977. Since then, spraying is done on the basis of Annual Parasite Index (API). HCH (50% wdp or 10% dust) is commonly used in agriculture. Besides HCH, cythion dust (5%) or furadon granules are also used. Liquid pesticide formulation (emulsion concentrate) included dimecron, dursban, malathion and sumithion.

Susceptibility of adult *Anopheles* was determined against 4% DDT and 0.4% dieldrin as per standard WHO procedure (Anon, 1981). Mosquitoes were exposed for one hour and mortalities recorded after a post-exposure period of 24 hours. All the tests were carried out under ambient temperature and relative humidity (A.T. 25-31°C, RH 68-89%), keeping control for each test.

Results of adult susceptibility tests are summarised in Table.1. It is evident from the data that *A.philippinensis* adults were highly susceptible to diagnostic dosages of DDT and dieldrin. The species was found susceptible to DDT in Meghalaya, Arunachal Pradesh and some areas of India, bordering Bangladesh (Rajagopal 1976, Nawab Singh and Chakraborty, 1979, Sundaram *et al.* 1980).

It is apparent from the results that besides indiscriminate use of pesticides in public health and agriculture, especially Tea Estates, so far there is no selection of resistance in *A.philippinensis* to DDT and dieldrin. This may be due to its exophilic nature and exophagous mode of feeding.

Accepted for publication: 14 January 1983.
[1] National Malaria Eradication Programme Zone, Silchar (Assam).
[2] Defence Research Laboratory, Tezpur-784001 (Assam).

Table 1— Susceptibility of *A. philippinensis* to DDT and Dieldrin in Assam*

Districts	PHCs	Period	DDT 4% concentration		Dieldrin 0.4% concentration	
			Number exposed	Percent mortality	Number exposed	Per cent mortality
CACHAR	Katlicherra	Jul '77	100	100	100	100
	Udarbond	Aug '77	30	100	-	-
	Sonai	Dec '77	50	100	35	100
	Lala	Jun '78	70	100	65	100
	Udarbond	Jul '78	70	100	60	100
	Sonai	-- do --	100	100	45	100
	Udarbond	Nov '78	55	100	40	100
	Jalalpur	May '79	60	100	55	100
	Borkhola	Nov '79	50	100	50	100
	Jalalpur	Mar '80	35	100	40	100
	Nilambazar	Jul '80	50	100	45	100
NORTH CACHAR HILLS	Gungunj	Sep '77	70	100	20	100
	do	Aug '78	30	100	35	100
	Langting	Sep '78	-	-	40	100
KARBI ANGLONG	Bokajan	Jun '77	30	100		--
	Manja	Mar '79	30	100	35	100
	Bokajan	May '80	45	100	50	100

* Tests where control mortalities were 20% or slightly up, have been discarded.

REFERENCES

1. Anonymous (1981). Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorine, organophosphate and carbamate insecticides. Diagnostic test. WHO/VBC/81.805. (Mimeographed document).
2. Nawab Singh and S.C. Chakraborty (1979). Susceptibility of *Anopheles philippinensis* to DDT from some areas of India bordering Bangladesh. *J. Com. Dis.* **11(2)**: 85-88.
3. Rajagopal, R. (1976). Studies on persistent transmission of malaria in Burnihat, Meghalaya. *J. Com. Dis.* **8(4)**: 235-245.
4. Sundaram, R.M., A.K. Sen, V.M. John, and P.K. Chakraborty (1980). A note on DDT susceptibility status of some *Anopheles* mosquitoes in Arunachal Pradesh. *J. Com. Dis.* **12(4)**: 210-211.

Indian Journal of Malariology
Vol. 19, Dec. 1982, pp. 147-150

A Double Headed *A. culicifacies*

S. RAMASESHA NARAYANA and P. GOPALAN

INTRODUCTION

Anomalies noted so far among anophelines are mainly related to the changes in number, colour, size and banding of different external appendages (genetic anomalies) (Rajagopal and Chakravarthy, 1960; Wattal *et al.* 1960, Wright and Pal, 1967). The lone morphological abnormality reported so far amongst the *Anophelines* is the existence of gynandromorphism (Manson, 1980). This note deals with the unique occurrence of a double headed *A. culicifacies*, which is the first report of existence of such an anomaly in the members of the family Culicidae (WHO, 1963).

A team from the Regional Office for Health and Family Welfare, Bangalore, had undertaken routine entomological collections in Kolar District, Karnataka State, for identifying the vectors of malaria and of Japanese Encephalitis, during September 1981. In one of their collections made near Ganjigunte Primary Health Unit in Sidlaghatta Primary Health Centre, a female *A.culicifacies* with two heads was noticed. This peculiar anatomical feature was observed while preparing the mosquito for dissection for sporozoites. During this particular study, a total of 735 *A.culicifacies* was collected out of which 221 were taken up for dissection.

Accepted for publication 15 January 1983.
Regional Office for Health & Family Welfare, Bangalore.

Specimen description (Fig. 1): The specimen under study was a live, fully fed, female *A.culicifacies* (Puri, 1938) with two heads. Each head had separate sets of appendages. The two heads were similar in size and shape with perfect bilateral symmetry. The heads were placed one below the other in such a position that they appeared like mirror images. Head-1 was in the natural position of attachment to the thorax while Head-2 was placed ventral to Head-1. The two heads were facing each other.

There was a single thorax and a single, but a larger abdomen. A seventh limb-bud was noticed arising from the right lateral aspect of the first abdominal segment. The legs and wings were typical of *A.culicifacies*. The details of the appendages are given in Table-1.

The specimen had taken a blood meal but it is not clear from which head.

The specimen was killed and mounted on a microscopic slide in Canadabalsum, but later it was remounted in DPX.

ACKNOWLEDGEMENT

The authors are also thankful to Messrs. V. Balasubramaniam and Y. Augustine Arumapandy, Senior Technicians of the Regional Office for Health and Family Welfare, Bangalore for their kind assistance on the field.

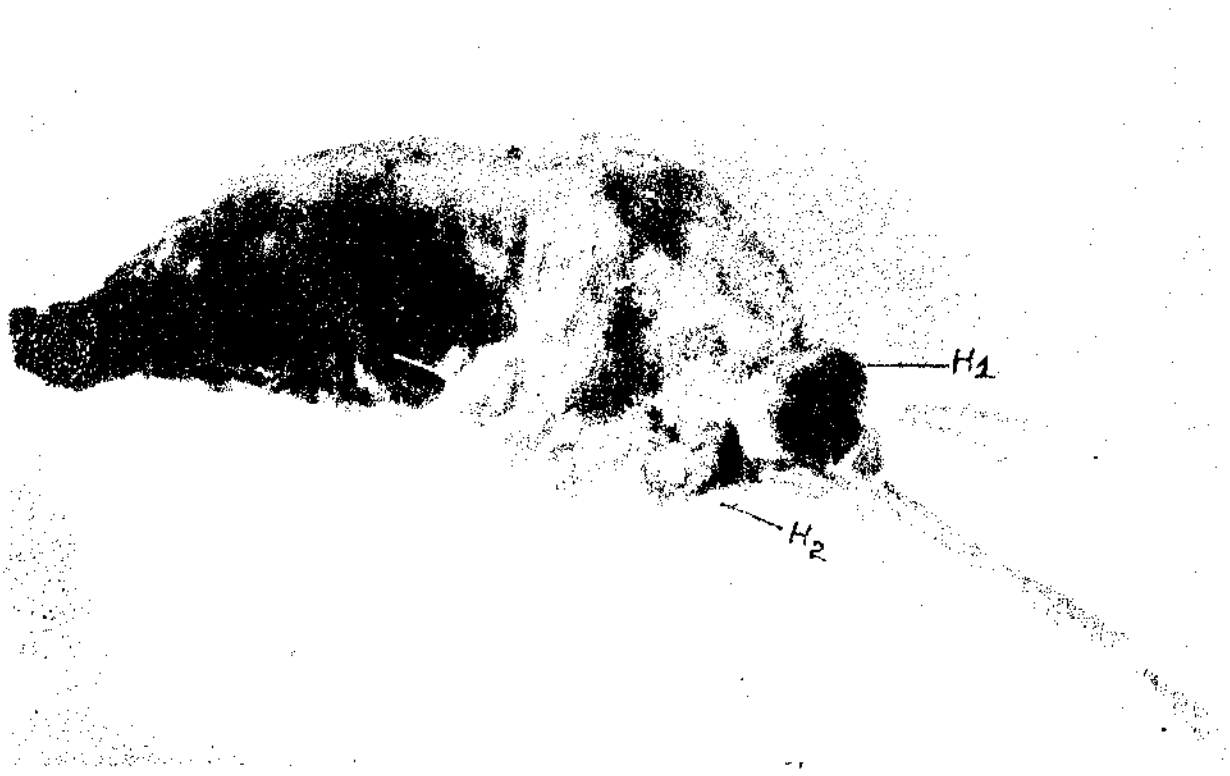


Fig. 1 Whole specimen of *A cuticifacies* showing two heads with separate mouth parts. (H_1 -Head one—normally situated head. H_2 Head two ventrally situated head).

Table 1 – Description of the specimen.

	Particulars	Head – 1	Head – 2
1.	Origin of the Head	From the Anterodorsal aspect of the thorax (normally situated).	From the Anteroventral aspect of the prothoracic region.
2.	Neck	Distinctly seen.	Embedded in the soft tissues of the thorax and is present between the anterior pair of legs.
3.	Palps		
	a) Type	Female	Female
	b) Segments	Three	Three
	c) Banding	Typical of *A. culicifacies*	Typical of *A. culicifacies*
4.	Proboscis	Both the proboscis were of equal length	
5.	Antennae		
	a) Type	Female (short haired type)	Female (short haired type)
	b) Segments	Right 9 segments Left 14 segments	Right 9 segments Left 14 segments
6.	Compound eyes	Normal	Normal

Note: Head-1 was in the normal head position while Head-2 was placed ventrally to Head-1 in the mirror image position.

REFERENCES

1. Manson, C.F. (1980). "A gynandromorph in *Anopheles gambiae*". *Mosquito News*. Vol. 40, No. 1, pp. 104-106.
2. Puri, I.M. (1938). "Synoptic table for the identification of Anopheline mosquitoes of India" (3rd Ed.). Health Bulletin No. 19, Manager of Publications, Delhi, pp. 21, 51.
3. Rajagopal, R. and R.K. Chakravarthy, (1960). "Records of Morphological variations in *A. annularis* Van der Wulp, near Dhanbad". *Ind. Jour. Mal.* 14: 2, pp. 171-174.
4. Wattal, B.L., N.L. Kalra, and M.L. Mammen, (1960). "Observations on certain morphological abnormalities in twenty species of Indian Anophelines". *Ind. Jour. Mal.* 14: 3, pp. 291-310.
5. WHO (1963). "Terminology of Malaria and of Malaria Eradication-Report of a Drafting Committee".
6. Wright, J.W. and R. Pal, (1967). "Genetics of Insect Vector of Diseases" (4th Ed.). Elsevier Publishing Company.

INDIAN JOURNAL OF MALARIOLOGY

Notes for Contributors

1. The Indian Journal of Malariology is devoted to the publications of original research which contributes significantly to the knowledge in any field of malariology. Papers of routine and repetitive nature dealing with gross observations may not be included.

2. Manuscripts should be sent in triplicate to the Editor, Indian Journal of Malariology, Malaria Research Centre, 22 Sham Nath Marg, Delhi 110054. Manuscript once sent to this journal implies that it is not under consideration for publication in any other journal.

3. Articles will be published at the editor's discretion in the order accepted. Date of acceptance will be that date on which copy is accepted in final form for publication.

4. The manuscript should be typewritten in English in double space on one side of the paper. Pages should be numbered consecutively. The matter should be arranged in the following order: Title, name(s) of the author(s) with address of the Institute, University (as foot notes and numbered serially in superscript), Abstract, Introduction, material and methods, results, discussion, acknowledgement, and references. Abstract, tables and captions of the figures should be typed on a separate sheet of paper. Line drawings should be clear and lettered in Indian ink. In case of photographs, good quality pictures should be provided. Both line drawings and photographs should be sent in duplicate, duly supplied with legends and separated from the text but allocated to sections of the text.

5. The International system of units (SI Units) should be used for all measurements. (Kindly see WHO publication, *The SI for the Health Professional*, WHO, Geneva 1977).

6. Author should provide a short title to be used as running title, of not more than 5-6 words in case the title of the paper is long.

7. References in the text may be cited as Sharma *et al.* (1976) or (Mount *et al.*, 1972; Coombes *et al.*, 1973; Mount *et al.*, 1974). The references to the paper accepted for publication, the word 'in press' should appear after the title of the periodical. The reference to the unpublished work should not be included in the list, it should be incorporated in the text itself (e.g., R.G. Roy, unpublished data or S. Pattanayak, personal communication). If the reference is to an article published without any authorship in a periodical, in place of author's name the term "Anonymous" should be used.

Title of Periodicals cited in the references are to be abbreviated as in the *World List of Scientific Periodicals*, 4th edition, 1964.

Reference of the literature, arranged alphabetically, and numbered consecutively, must be placed at the end of the paper. The following procedure may be followed.

Research Papers

Sharma, V.P. (1976). Elimination of aziridine residues from chemosterilised mosquitoes. *Nature*, **261** (5556): 135.

Ansari, M.A., V.P. Sharma and R.K. Razdan (1978). Mass rearing procedures for *Anopheles stephensi* Liston. *J. Com. Dis.* **10**(2): 125-135.

Books, Monographs

Rao, T.R. (1981). *Anophelines of India* (W.C). Indic Press, Bangalore).

Landau, I. and Y. Boulard (1978) In *Potent Malaria*, edited by K. Killick-Kendrick and W. Peters (Academic Press Inc., London) 43-84

Paper presented to Symposium/Conference

Subbarao, S.K. *Cytoplasmic incompatibility in mosquitoes.* Paper presented to the International symposium on recent developments in the genetics of insect diseases vectors, Bellagio, Italy, 20-24 April, 1981

8. Short notes should be prepared in a manner similar to the research papers and will have title, name(s) of author(s) with address of Institute/University as footnotes, acknowledgement and references.

9. Reviews would be published of the invited papers only. Book reviews may be published at the end of journal.

10. Galley proofs of the articles will be sent to the authors for correction. Corrected proofs must be returned promptly to the editor or else the article will not be printed in the current issue or may be printed as it stands.

11. Reprints will be mailed to the senior author. Twentyfive reprints will be supplied free of charge to single author and thirty for joint authors to be distributed on equal basis. Additional reprints may be ordered on payment when the proofs are returned. A reprint order form will be supplied with the proofs.

Note All correspondence regarding contribution and subscription should be addressed to the Editor, Indian Journal of Malariology, 22, Sham Nath Marg, Delhi-110 054.

INDIAN JOURNAL OF MALARIOLOGY

Authors Index

EDITORIAL ACKNOWLEDGEMENT

Editor gratefully acknowledges the help of following scientists who have kindly reviewed the papers for the 1982 issues of the Indian Journal of Malariology.

Dr. G.P. Dutta, Central Drug Research Institute, Lucknow; Dr. U.V. Wagh, National Institute of Virology, Pune; Dr. K.N. Mehrotra, Indian Agricultural Research Institute, New Delhi; Sh. B.S. Krishnamurthy, Munirka, New Delhi; Dr. (Mrs.) K. Jayaraman, Madurai University, Tamil Nadu; Dr. Ramesh Kumar, All India Institute of Medical Sciences, New Delhi; Dr. R.C. Mahajan, Postgraduate Institute of Medical Education & Research, Chandigarh; Dr. A. Gajanana, National Institute Communicable Diseases, Delhi; Dr. (Mrs.) Dipika Kaul, University of Alhabad, Allahabad; Dr. G.P. Talwar, All India Institute of Medical Sciences, New Delhi; Dr. A.N. Malaviya, All India Institute of Medical Sciences, New Delhi; Dr. S.D. Seth, All India Institute of Medical Sciences, New Delhi; Dr. Srinivas, All India Institute of Medical Sciences, New Delhi; Dr. B.B. Gaitonde, World Health House, New Delhi; Dr. N.N. Singh, Institute for Research in Medical Statistics, New Delhi; Dr. R.G. Roy, Central Leprosy Training Institute, Chingalpat, Tamil Nadu; Dr. S.K. Parida, Indian Council of Medical Research, New Delhi; Dr. (Mrs.) Shobha Sehgal, Postgraduate Institute of Medical Education & Research, Chandigarh; Dr. R. Nath, Postgraduate Institute of Medical Education & Research, Chandigarh; Dr. A.N. Rai Choudhury, National Institute of Communicable Diseases, Delhi; Dr. S.R.V. Rao, University of Delhi, Delhi; Dr. B.N. Chowdaiah, Bangalore; Dr. P.P. Gudadhe, Haffkin Institute, Bombay; Dr. Ramesh Kumar, All India Institute of Medical Sciences, New Delhi; Dr. D. Subramanyam, CIBA-GEIGY Research Centre, Bombay; Dr. A.B. Sen, Central Drug Research Institute, Lucknow; Dr. T.N. Ghosh, School of Tropical Medicine, Calcutta; Dr. Gauri Bazaz Malik, Lady Hardinge Medical College, New Delhi; Dr. B.L. Wattal, National Institute of Communicable Diseases, Delhi; Dr. C.K. Rao, National Institute of Communicable Diseases, Delhi; Dr. Sarala K. Subbarao, Malaria Research Centre, Delhi; Dr. D.S. Choudhury, Malaria Research Centre, Delhi; Dr. T. Ramachandra Rao, Malleswaram, Bangalore.

Statement about ownership and other particulars of
Indian Journal of Malariology

FORM IV

(See Rule 8)

1.	Place of Publication	Delhi
2.	Periodicity of its publication	Half yearly
3.	Printer's Name	V.P. Sharma
	Nationality	Indian
	Address	Malaria Research Centre 22 Sham Nath Marg, Delhi 110 054
4.	Publisher's Name	Dr. V.P. Sharma
	Nationality	Indian
	Address	Malaria Research Centre 22 Sham Nath Marg, Delhi 110 054
5.	Editor's Name	Dr. V.P. Sharma
	Nationality	Indian
	Address	Malaria Research Centre 22 Sham Nath Marg, Delhi 110 054
6.	Name and address of individuals who own the newspaper and partners or shareholders holding more than one per cent of the total capital.	Does not arise

I, V.P. Sharma, hereby declare that the particulars given above are true to the best of my knowledge and belief.

Sd -
V.P. Sharma
Signature of Publisher

9 March 1982