An *In Vitro* Approach to Study Innate Resistance to *Plasmodium falciparum* Infection in Folic Acid Deficient Individuals*

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SUMMARY *Plasmodium falciparum* **was cultured in folic acid deficient erythrocytes using the** *in vitro* **micro-technique and macro-technique. We found that the parasites could not undergo a complete schizogony when folic acid in the culture medium was kept at a deficient level of 1 ng/ml. Results from this study suggested a natural resistance to malaria infection in folic acid deficient individuals, and they confirmed the requirement for folic acid by sulfadoxine resistant** *P. falciparum.*

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Malaria parasites are believed to lack the enzyme folate reductase and thus to be unable to utilize preformed folic acid.[1] Studies performed on rodent malaria *Plasmodium berghei* suggested a parasite requirement for para-aminobenzoic acid (PABA) as a substrate for the biosynthesis of folate cofactor. This biosynthetic pathway occurs among many bacteria.[2] These findings have had an impact on the development of antimalarials. For example, sulfonamides, competitive antagonists to PABA, and pyrimethamine, an inhibitor of dihydrofolate reductase are used for the treatment of malaria. The efficacy of this drug combination was 96 per cent in 1970.[3] However, since then malaria parasites have developed resistance to both compounds of either group rapidly.[4] Our recent studies on mechanisms of drug resistance in *P. falciparum* suggested that, once the parasites became resistant to sulfadoxine they no longer used PABA for dihydrofolate biosynthesis[5] but that they used folic acid[6] instead.

Generally, folic acid concentrations in human serum are between 3-20 ng/ml depending on diet, intestinal flora, and absorption capacity of the intestinal mucosa. A folic acid value below 2 ng/ml is considered symptomatic of deficiency and is recognized by signs of anaemia.[7] It was the purpose of this work to examine whether this type of haematologic disorder could offer any protection against malarial infection similar to that already recognized in thalassaemia, sickle-cell anaemia and glucose 6-phosphate dehydrogenase (G6PD) deficient anaemia.

MATERIALS AND METHODS

Plasmodium falciparum lines NT-21 and NT-22 were isolated from patients at the malaria clinic (Tak province) of the Ministry of Public Health. The parasites have been maintained in continuous culture[8] since August 1983. Tests for drug responsiveness revealed that NT-21 was moderately sensitive to pyrimethamine and sulfadoxine, whereas NT-22 was resistant to both.

Culture medium for routine culture was RPMI 1640 (GIBCO) containing 25 mM HEPES, 0.2% $NaHCO_3$ and 10% human serum by volume. For experiments requiring controlled concentrations of folic acid, RPMI special formula (GIBCO) which contained neither folic acid nor PABA was used. The medium was reconstituted in the same manner as RPMI 1640. It was designated as RPS. After one millilitre of pooled serum which contained 10 ng/ml folic acid was added to 9 millilitres of RPS, the final folic acid

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concentration of RPS + human serum was thus not higher than a deficient level of 2 ng/ml.

Erythrocytes were from two individuals: a healthy donor who had blood group O (designated N-cells) and a folic acid deficient donor. From the latter, anaemia due to folic acid deficiency was diagnosed using clinical signs and laboratory findings. Erythrocyte morphology was typically macrocytic and normochromic, and the reticulocyte production index was 5-6 per cent. After 10 millilitres of blood had been taken and stored aseptically in acid citrate dextrose (ACD), the patient was given a folic acid therapy. After 15 days the patient recovered from anaemia. This further confirmed that the diagnosis of folic acid deficiency was correct. The abnormal erythrocytes, designated as D-cells in this report, were then used for experiments.

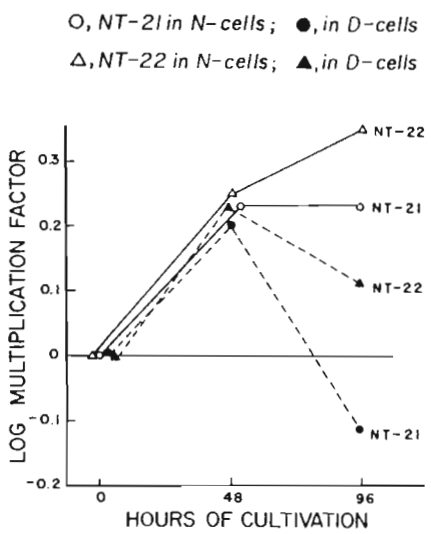


Fig. 1 Population growth of *Plasmodium falciparum* cultured in RPMI 1640.

Experiments. Parasite growth in normal and folic acid deficient cells was compared in replicated glass-covered vials (2 x 3 cm), each containing 0.9 millilitres of RPMI 1640 culture medium, i.e., by the macrotechnique. Stock cultures of *P. falciparum* harbouring late schizonts with parasitaemia of 9-10 per cent were pooled and centrifuged to make a 50 per cent suspension. This was mixed with freshly washed N-cells or D-cells (in 50% suspension) so that the parasitaemia was 1 per cent. These parasite-blood cell suspensions were dispensed in 0.06-ml volumes into the prepared vials and incubated in a candle-jar atmosphere at 37.5°C. Culture medium was changed at 48 hours and 96 hours, at which time a small sample was taken from each vial to make thin blood smears.

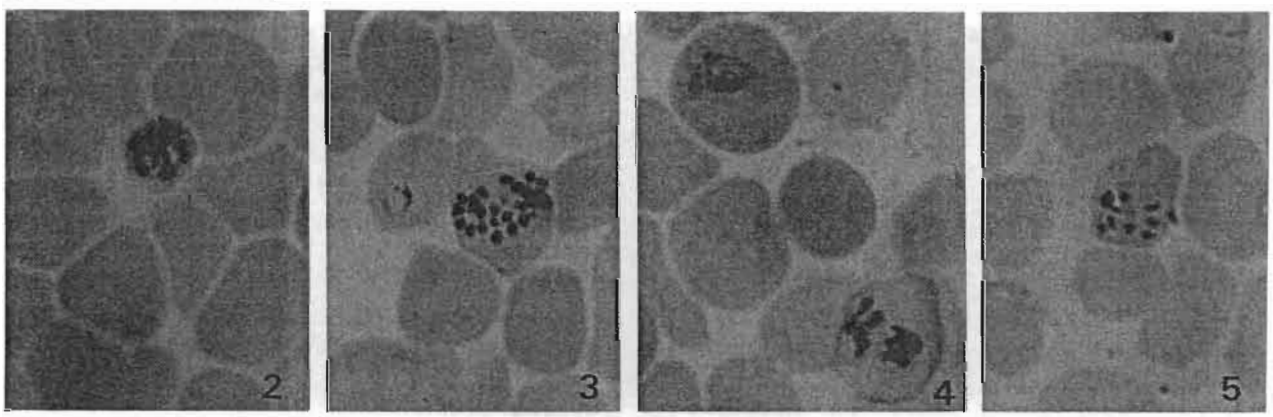
In the experiments where the folic acid concentration was kept at a deficient level[7] parasite-blood suspensions were prepared as described above. They were further diluted with RPS + human serum to contain 6 per cent haematocrit, and dispensed in 50- μl volumes into flat-bottomed wells of a microtiter plate with 4 replicates for each variable. The microcultures were evaluated after 48 hours in order to avoid cell lysis, which was sometimes found in abnormal erythrocytes when they were cultured as long as 96 hours (Brockelman, unpublished data).

To evaluate the suppressive effects of D-cells, parasites were counted directly in thin blood films made at 0 hour, 48 hours and 96 hours. The *in vitro* growth of the parasite was exponential during this interval. The logarithm of the parasite multiplication factor (per one schizogony, i.e., 48 hours) therefore provided a measure of the exponential rate of parasite growth[9] and was used in the test of multiple comparison of the effects of cell types. Factorial analysis of two factors was carried out to test the effects of cells and media.[10]

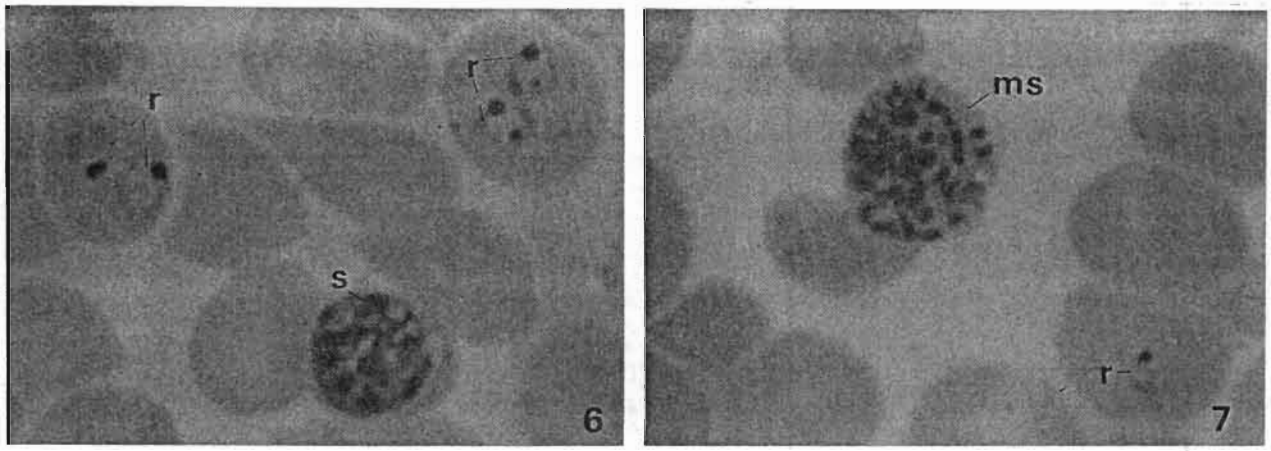
RESULTS

Cultures in RPMI 1640

The *in vitro* growth of *P. falciparum* in folic acid deficient erythrocytes is summarized in Fig. 1. It is apparent that there were no differences in multiplication factors



Figs. 2-5 Plasmodium falciparum cultured in folic acid deficient medium (RPS). 2, a mature schizont of N-22 in an N-cell after 48 hours of cultivation. 3, a 96-h culture in D-cells, right, a mature schizont in a macrocytic erythrocyte; left, a late ring stage. 4-5, *P. falciparum* in RPS and D-cells, 4, upperleft, an ill-looking trophozoite; low right, a macrocytic erythrocyte harbouring two deformed schizonts. 5, An abnormal segmenter in a D-cell.



Figs. 6-7 Plasmodium falciparum in control cultures. The parasites are cultured in normal erythrocytes and RPMI medium. 6, a healthy schizont (s) and four healthy rings (r); 7, an erythrocyte harbouring two mature schizonts (ms).

during the first 48 hours of cultivation. D-cells could support growth of both culture lines as did the N-cells which served as controls (Fig. 2). Impairment of growth, however, could be detected when the cultivation time was extended to 96 hours. It could be seen on the thin smears stained with Giemsa that development of the re-invaded merozoites was interrupted resulting in ill-looking schizonts (Fig. 3). Quantitative counts of parasites against 5,000 erythrocytes revealed a marked difference in the number of parasites when grown in N-cells or D-cells. In the latter's the NT-22 population declined whereas it increased in N-cells. The NT-21 population also increased at the same rate in N-cells throughout the 96 hours of cultivation, but growth stopped completely in D-cells. A multiple comparison test showed a significant difference among the mean multiplication factors ($P < 0.01$) for both the N-21 and N-22 culture lines when grown in N- and D-cells for 96 hours.

Cultures in RPMI special formula (RPS)

Differences in development of *P. falciparum* in N-cells and D-cells could be recognized readily by parasite morphology after 48 hours of cultivation. As shown in Figs. 4 & 5 merozoites which invaded the D-cells developed poorly. Effects of folic acid deficiency could be seen directly from the parasite nucleus which failed to enlarge proportionally to cytoplasmic expansion. Although nuclear division could be observed occasionally (Fig. 5), it did not give rise to invasive merozoites. Hence, merozoite invasion into new cells did not occur, and this resulted in a decline in parasite population. At the same time, schizonts of both NT-21 and NT-22 in normal erythrocytes (N-cells) became mature and ruptured, and the merozoites re-invaded neighbouring erythrocytes (Figs. 6 & 7).

The effects of cells and media were demonstrated quantitatively by the factorial-designed experiments. Results summarized in Tables 1 and 3 clearly show significant interactions between the cells and the media. Suppression of parasite growth was most pronounced when both culture lines were grown in D-cells in folic acid deficient medium. There was a significant reduction in the multiplication factor when NT-21 was cultured in D-cells in RPS (Tables 1 and 2). However, its multiplication did not differ from that in N-cells when it was cultured in RPMI. Results from

Table 1 Effects of folic acid deficient cells and culture media on multiplication factors of *Plasmodium falciparum* NT-21 (four replicates).

Factor A (Cells)	Factor B (Media)	
	RPMI	*RPS*
N – cells	0.328	0.360
	0.364	0.372
	0.321	0.241
	0.338	0.324
D – cells	0.420	0.172
	0.481	0.131
	0.420	0.082
	0.490	0.128

The combination RPMI, N-cells represents cultivation of *P. falciparum* in normal cells in RPMI medium, etc. Significant effects of interaction are shown in Table 2.

Table 2 Analysis of variance shows interactions of folic acid deficient cells and culture media which inhibited growth of *Plasmodium falciparum* line NT-21.

Factors	Degree of freedom	Sum. square	Mean squares	F	P
Cells	1	0.007	0.007	3.5	0.05
Medium	1	0.115	0.115	57.5	0.001
Cells x medium	1	0.096	0.096	48.0	0.001
Error	12	0.020	0.002		
Total	15	0.238			

$F_{.01\ (1,12)} = 9.33$
$F_{.05\ (1,12)} = 4.75$

Table 3 Interactions of folic acid deficient cells (row) and culture medium (column) on multiplication of *Plasmodium falciparum* line NT-22. Each number represents common logarithm of multiplication factor (log MF). Negative values indicate population decline.

Factor A (Cells)	Factor B (Media)	
	RPMI	*RPS*
N – cells	0.385	0.212
	0.401	0.260
	0.334	0.209
	0.440	0.220
D – cells	0.155	– 0.262
	0.203	– 0.140
	0.204	– 0.021
	0.041	– 0.541

this experiment coincided well with those from the macro-method presented in Fig. 1.

Additive effects of medium and folic acid deficient cells were more marked in experiments using culture line NT-22. The parasite multiplication factor was reduced to as low as -0.541, i.e., there was a decline in parasite population with the combination of D-cells and RPS medium (Table 3).

DISCUSSION

Folic acid deficiency has been included as one of the vitamin deficiencies contributing to malnutrition problems in Thailand. Its role in malaria infection in endemic areas has long been questioned.[10] The study reported herein is therefore of significant interest for several reasons. The *in vitro* cultivation techniques decribed by Trager and Jensen[8] allows direct examination of *P. falciparum* responses to substances or factors in a clean system, where interferences by host factors are excluded. Nevertheless, one must be fully aware that the culture medium RPMI 1640 was originally designed to meet *in vitro* requirements of leukaemic cells[11] and thus contains vitamins and co-factors in excessively high concentrations. Brockelman and Tan-ariya[12] have demonstrated that PABA in RPMI is dispensable, and that its presence at that high concentration (1,000 ng/ml) antagonizes *in vitro* sulfonamide activities. Similarly, the folic acid concentration in RPMI is too high (1,000 ng/ml). *In vitro* examination of folic acid deficient erythrocytes with regard to supporting parasite growth would have been meaningless if the folic acid concentration in the test medium had not been controlled within the deficiency range, i.e., to represent the value found *in vivo*. The use of RPMI special formula allowed us to examine parasite growth without competition of folic acid in the culture medium.

The *in vitro* micro-technique used in this study was modified from the technique developed by Rieckmann[13] to assess *P. falciparum* sensitivities to chloroquine. The method proved applicable to other studies for which large blood volumes could not be obtained. It would be unethical to take a blood sample larger than 10 millilitres from patients suffering from anaemia.

This study further confirms our previous observations[6] that sulfadoxine sensitive parasites used PABA for the biosynthesis of folate cofactor, whereas the resistant ones prefer folic acid from an exogenous source. *P. falciparum* line NT-21 was less resistant to sulfadoxine, and this drug response characteristic was expressed. When folic acid was depleted from the medium (RPS) the parasites were not affected as much as were those of line NT-22 which was sulfadoxine resistant. NT-22 depended more on folic acid in the medium, since it had already ceased to grow after 48 hours in RPS.

Innate resistance to *P. falciparum* infection in man is well recognized *in vivo* and it had also been demonstrated *in vitro*. At least three types of erythrocyte abnormalities have been studied with results showing a significant suppression in parasite multiplication. Epidemiological studies in Thailand[14] on the role of haemoglobin E in protecting against malaria have been confirmed *in vitro*.[15] Like-

wise, sickle-cell genes protect heterozygous individuals against the lethal effects of malaria and the mechanism of that protection has been studied *in vitro*.[16] A third type of red blood cell abnormality is glucose-6-phosphate dehydrogenase (G6PD) deficiency. Quantitative evidence that this deficiency is the basis for genetic determinants of relative resistance has been shown by well planned epidemiological study,[17] and *in vitro* studies are now being persued.[18] Results of the experiments reported herein show an impairment of growth of *P. falciparum* in folic acid deficient erythrocytes, a phenomenon which possibly gives relative protection against malarial infection *in vivo*.

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