PLASMODIUM FALCIPARUM STRAINS FROM PAPUA NEW GUINEA: CULTURE CHARACTERISTICS AND DRUG SENSITIVITY

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INTRODUCTION

Malaria is hyperendemic in the coastal regions of Papua New Guinea while the highlands are either malaria-free or subject to epidemic malaria. The prevalent species are *Plasmodium falciparum*, *P. vivax* and *P. malariae*. Chloroquine resistant *P. falciparum* are present (Han, 1978) and the frequency is increasing (Vrbova and Gibson, 1980). People in hyperendemic areas of Papua New Guinea exhibit high levels of malarial antibodies (Curtain *et al.*, 1964) and marked splenomegaly is present in some groups (Crane, 1972).

The genetically diverse population includes high frequencies of mutations which may relate to the high incidence of malaria (Kidson, 1961; Curtain *et al.*, 1962., 1965; Serjeantson *et al.*, 1977), suggesting that malaria may have exerted an important selection pressure over a long time period.

Since *P. falciparum* was first cultured continuously *in vitro* (Trager and Jensen, 1976), several other strains of this malaria parasite have been established (Jensen and Trager, 1978). The early cultures were derived from parasites passaged through *Aotus trivirgatus* monkeys but later strains were grown directly from infected human blood. However, not all samples of infected blood yield a continuous line (Jensen and Trager, 1978). Presently, there is considerable interest in the feasibility of developing malaria vaccines. However, little is known of antigenic variation between different strains of *P. falciparum*. Further continuous lines from different malarious regions in the world are needed to study antigenic variation and other properties such as mechanisms of drug resistance. We report here the establishment of a series of *P. falciparum* strains from Papua New Guinea in long-term culture, together with a description on their culture characteristics and assessment of their drug sensitivity status.

MATERIALS AND METHODS

Parasites: Venous blood was collected aseptically into bottles containing heparin from 37 patients with clinical malaria in Madang Hospital, Madang Province, Papua New Guinea. The cells were washed twice in culture medium lacking serum and resuspended to 10% v/v in medium for transportation on wet ice by air to Brisbane or Melbourne where they were cultured after 1 to 4 days in transit. Nine further samples were collected from patients in Brisbane whose infections were acquired in Papua New Guinea and these were cultured as soon as the laboratory was notified. Parasite densities were recorded from smears made at the time of receipt.

Culture: The method used for parasite culture was essentially that of Trager and Jensen (1976). Parasites were grown in

human type AB (or in some cases type A) erythrocytes in RPMI-1640 medium (Commonwealth Serum Laboratories, Melbourne) supplemented with 24 mM HEPES buffer (Calbiochem, San Diego), 20 µg/ml gentamicin sulfate (Sigma Chemical Co., St Louis), and 10% human type AB (or A where relevant) serum. The pH was adjusted to 7.4 with $NaHCO_3$. Erythrocytes and serum were obtained by the kind cooperation of the Red Cross Blood Bank, Brisbane and donors from the Royal Melbourne Hospital. Parasites were subcultured twice weekly to maintain parasitaemia between 0.5% and 4%.

Storage of continuous lines: Parasitized erythrocytes were stored in liquid nitrogen as described by Rowe *et al.*, (1968).

Chloroquine sensitivity: Assay of parasite sensitivity to chloroquine diphosphate was carried out by the micromethod of Rieckmann *et al.*, (1978). In selected cases chloroquine sensitivity was quantified on samples at the time of collection and in some of these sensitivity was again tested on the same parasite strain in long-term culture. In Madang tests were done using 5 µl of fresh blood added to each well of microtitre plates containing freshly prepared solutions of drug in medium. Cultured strains were first synchronized by using 0.75% swine skin gelatin (Jensen, 1978)or sorbitol lysis (Lambros and Vanderberg, 1979) to select ring stage parasites which were then added to the microtitre wells. In each set of assays two control wells without drugs were included. All laboratory parasite strains were tested at least twice.

Pyrimethamine sensitivity: The established lines were assayed for sensitivity to pyrimethamine. A 10 mM solution was obtained by dissolving pyrimethamine (Burroughs Wellcome & Co., Rosebery), in 0.5% lactic acid (Richards and Maples, 1979). Dilutions were made in culture medium and 25 µl aliquots were added to microtitre plates. Fresh solutions were made prior to each experiment. Lactic acid in the absence of pyrimethamine did not inhibit parasite development at the lowest dilution. After selection for ring forms with 5% sorbitol, the cells were resuspended to 10% (v/v) in culture medium and 25 µl of this suspension were added to each well. Three wells without pyrimethamine were included as controls. The plates were incubated in a humidified candle jar at 37°C for 24 hours. As the leucocytes had essentially been removed from the blood used in culturing, obviating their use for reference, approximately 10^4 nucleated goose erythrocytes were mixed with the contents of each well for this purpose prior to making smears. Thick films were made from each well and stained with 2% Giemsa. The number of schizonts with 3 or more nuclei per 100 goose erythrocyte nuclei was determined and the result compared with the mean of the three control wells. All strains were tested at least twice.

RESULTS

Establishment of continuous lines: Forty-six samples of parasitized blood were received of which 44 were cultured and of these 7 have been in continuous culture for more than 12 months (Table 1). Generally samples with

Table 1

Survival of cultured *P. falciparum* strains.

Source	No. Samples	No. Cultured	*Survival in culture*	
			>1 month	>12 months
Madang	37	35	12	6
Brisbane	9	9	3	1

higher parasitaemias survived longer. However not all samples with a high parasitaemia formed continuous lines and neither did any sample in transit for more than 3 days. The age of the donor did not affect the development of a line (Table 2). Parasites in some cultures immediately underwent gametogenesis and asexual forms were not seen after 2-3 days in culture. Other strains underwent several intra-erythrocytic cycles before parasite numbers inexplicably fell. Some cultures were lost when a particular batch of erythrocytes or serum did not support growth. Although 6 of the 7 continuous lines were found to be chloroquine resistant, resistance did not necessarily lead to a continuous line as attempts to culture 5 chloroquine resistant samples, all with high initial parasitaemias were unsuccessful. Samples of all the continuous lines were stored in liquid nitrogen after cultures were established and were recoverable from the frozen state for later culture.

Chloroquine sensitivity: The continuous *P. falciparum* lines were assayed for chloroquine sensitivity and the results are shown in Table 3. Growth in the well containing 1 nmol chloroquine diphosphate/ml of blood indicates resistance (Antunano and Wernsdorfer, 1979). Six of the lines were resistant,

Table 2

Collection characteristics of long-term surviving strains.

Strain	Age of Donor	Initial Parasitaemia (%)	Days in Transit at 4°C
FCQ-2/PNG	5	2.3	0
FCQ-22/PNG	15	2.0	2
FCQ-27/PNG	4	6.0	3
FCQ-30/PNG	30	10.0	1
FCQ-31/PNG	5	2.5	2
FCQ-33/PNG	2	3.0	2
FCQ-41/PNG	19	1.0	2

Table 3

Chloroquine sensitivity of parasite strains surviving in long-term culture.

Strain	% Schizonts compared with controls. Concentration of chloroquine diphosphate (nmol/ml blood)										Drug* status
	0	0.2	0.4	1.0	2.0	4.0	10.0	20.0	40.0	100.0	
FCQ-2/PNG	100	88	106	69	44	50	13	0	0	0	R
FCQ-22/PNG	100	96	96	92	66	14	0	0	0	0	R
FCQ-27/PNG	100	103	91	79	62	12	0	0	0	0	R
FCQ-30/PNG	100	96	24	3	0	0	0	0	0	0	S
FCQ-31/PNG	100	90	60	22	2	0	0	0	0	0	R
FCQ-33/PNG	100	104	104	72	15	9	5	0	0	0	R
FCQ-41/PNG	100	105	107	71	17	2	2	1	0	0	R

* R = Resistant. S = Sensitive.

Table 4

Pyrimethamine sensitivity of cultured *P. falciparum* strains.

Strain	% Schizonts compared with controls. Concentration of Pyrimethamine (μM)									
	0	1.0	2.5	5.0	10.0	25.0	50.0	100	250	500
FCQ-2/PNG	100	141	126	120	78	67	28	15	0	0
FCQ-22/PNG	100	81	83	60	58	4	3	0	0	0
FCQ-27/PNG	100	93	95	88	84	33	4	0	0	0
FCQ-30/PNG	100	110	101	94	41	12	5	0	0	0
FCQ-31/PNG	100	132	157	128	56	23	4	0	0	0
FCQ-33/PNG	100	76	58	56	31	2	0	0	0	0
FCQ-41/PNG	100	75	92	84	117	22	2	0	0	0

and one, FCQ-30/PNG was partially sensitive. Other *P. falciparum* strains that did not form continuous lines or were not cultured were tested in Madang and 9/12 (75%) were determined to be resistant.

Pyrimethamine sensitivity: The continuous *P. falciparum* were assayed for sensitivity to pyrimethamine and the results are shown in Table 4. All strains showed $\geq 30\%$ survival at 10^{-5} M pyrimethamine as judged by schizont formation. This is about 3 orders of magnitude greater survival than that reported for an African strain using a somewhat different method of assay (Richards and Maples, 1979). By this criterion all the Papua New Guinea continuous lines are pyrimethamine resistant. None of the patients from whom these parasites were isolated had been treated with pyrimethamine.

DISCUSSION

Seven strains of *P. falciparum* from Papua New Guinea have been established in continuous *in vitro* culture. A significant proportion of the samples cultured did not form continuous lines due to formation of gametocytes or parasite death. Reliable histories of malaria prophylaxis were often unavailable and it is possible that, in some cases, antimalarials had been taken prior to collection of the blood. This could explain early lack of *in vitro* growth. The time between collection and culture was important and no sample cultured more than 3 days after collection formed a continuous line. The initial parasitaemia was also important and samples with a high parasitaemia were more likely to form a continuous line : no samples with $< 1\%$ parasitaemia formed lines. The age of the donor which conceivably could relate to immune status in the case of donors living continuously in the hyperendemic area had no apparent effect on the ability of a strain to form a line.

Chloroquine resistant *P. falciparum* are now prevalent in Papua New Guinea (Han, 1978) and this is reflected in the present *in vitro* chloroquine sensitivity assays. Since the proportion of chloroquine resistant strains established in continuous culture was similar to that in the general population of parasite strains in the region, no conclusions can be drawn about any possible relationship between drug resistance and establishment of lines tested in Madang. The marked difference in sensitivity to pyrimethamine of all sevenPapua New Guinea strains and of an African strain (Richards and Maples, 1979) is intriguing. The practical significance of the *in vitro* resistance to pyrimethamine is not yet known

although clinical resistance to Fansidar (pyrimethamine and sulfadoxine) has recently appeared in Papua New Guinea (Darlow *et al.*, unpublished data) and has been reported in neighbouring Irian Jaya (Rumans *et al.*, 1979).

Clearly, there are differences among strains from Papua New Guinea,including the ability to form continuous *in vitro* lines and drug sensitivity. Antigenic variation, an important consideration with respect to potential vaccines has yet to be studied although differences in antigenic composition of soluble products of three of the above lines have already been documented (Brown, unpublished observations). The establishment of continuous lines from Papua New Guinea provides a valuable source of material for detailed antigenic analysis and will enable comparison between *P. falciparum* from this region and other malarious regions in the world.

SUMMARY

Seven strains of *Plasmodium falciparum* from Papua New Guinea have been established in continuous *in vitro* culture. Samples with a high initial parasitaemia were more likely to form continuous lines, possibly due to the time required for transport of infected blood samples from Papua New Guinea to laboratories in Australia. Most but not all established lines were resistant to chloroquine and all were resistant to pyrimethamine, possibly reflecting the parasite strain characteristics in that region.

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