Plasmodium falciparum in continuous culture: a new medium for the \textit{in vitro} test for sulfadoxine sensitivity*

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The sulfadoxine sensitivity of two strains of Plasmodium falciparum from Thailand, FCM2 and FCM5, was assessed using two types of culture medium, Waymouth formula and RPMI 1640. Growth of the parasite was completely inhibited by 0.5 mmol of sulfadoxine per litre of Waymouth formula, whereas parasite growth in RPMI was not affected at this concentration. The apparent difference in drug sensitivity was shown to be caused by competition between 4-aminobenzoic acid and sulfadoxine. This hypothesis was further confirmed by the extent to which $^{14}$C-sulfadoxine was incorporated into the infected erythrocytes.

The recent development of a method for continuous cultivation of \textit{Plasmodium falciparum} in the laboratory (1) has permitted the study of the drug response of erythrocytic forms \textit{in vitro} over a test period of more than one developmental cycle. The results obtained with chloroquine and pyrimethamine have shown good agreement with \textit{in vivo} test methods (2, 3), but tests on sulfadoxine have required an unrealistically high concentration of the drug for minimal inhibition \textit{in vitro}, although the parasite showed high \textit{in vivo} susceptibility to sulfadoxine treatment. It has been suggested that this inconsistency is a result of competition between 4-aminobenzoic acid (PABA), a component of RPMI culture medium, and sulfadoxine, a PABA analogue (3). It was thus necessary to find a medium that supports parasite growth but contains no PABA. Such a medium would permit further screening of sulfa drugs, which have become increasingly important in South-East Asia where chloroquine-resistant \textit{P. falciparum} is now developing resistance to pyrimethamine (3, 4).

MATERIALS AND METHODS

Parasites

The \textit{P. falciparum} lines used in this study, FCM2 and FCM5, were isolated from two residents of Trat, south-east Thailand, in September 1980, and have since been kept in continuous culture in type O human erythrocytes in a Petri dish and candle jar system, as described by Trager & Jensen (1). Repeated experiments over a 3-month period showed that FCM2 was completely inhibited by 4 mmol and FCM5 by 12 mmol of sulfadoxine per litre of RPMI (3).

Culture media

The culture media used in this study were RPMI 1640 containing 1 mg of PABA per litre (5), and Waymouth powder formula which contained no PABA (5). Each medium was constituted to contain 25 mmol of HEPES, 2 g of NaHCO$_3$, and 100 ml of human serum per litre, the serum being collected from one individual. These media are designated complete RPMI (referred to as RP) and complete Waymouth (WM). In one series of experiments, 1 mg of PABA was added per litre of Waymouth, and used as a positive control.

Determination of PABA level

Human serum and complete media containing serum were deproteinized (6) and aliquots of 2 ml were reacted with 4 ml of sodium nitrite solution (1 g/litre) and 0.4 ml of ammonium sulfamate solution (5 g/litre). The mixture was coupled with 0.2 ml of an aqueous solution of \textit{N}-naphthylethylenediamine dihydrochloride (1 ml/litre). The optical density of the resultant colour was measured in a Unicam spectrophotometer at 545 nm. Mixtures without the coupling reagent served as a control. The standard PABA solution was made up as described by Howitz (7).

\begin{footnotesize}
\begin{itemize}
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\item a Both culture media were obtained from Grand Island Biological Company, New York, USA.
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\end{itemize}
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Response to sulfadoxine

The response of the parasites to sulfadoxine was assessed by a Petri dish test, as described by Trager et al. (8), and by a vial test (9). Parasites were grown in RP in 60-mm Petri dishes for 4 days, when a 4% parasitaemia was reached, then pooled and washed repeatedly in WM. A total of 64 subcultures were made in 35-mm Petri dishes, 32 of which received WM medium and 32 RP. Cultures were maintained without drug for 2 days and then exposed for 2 days to sulfadoxine dissolved in the appropriate medium at a concentration of 0, 0.5, 1, 2, 4, 6, 8, or 10 mmol/litre (prepared from a 1 mol/litre solution of sulfadoxine). Parasite counts per 10 000 erythrocytes were made at the beginning of the experiment, before exposure, and after exposure. Parasite population rates of change during the 2 days of drug treatment were expressed as the common logarithm of the parasite multiplication factor (9).

To study the incorporation of [14C]-sulfadoxine, 4-day old cultures were centrifuged at 250 g to concentrate the erythrocytes harbouring ring forms. The dark top layer was discarded and the remaining packed cells were subcultured in RP or WM. An initial parasitaemia of 1% increased to 10% (80% trophozoites) within 3 days in RP or WM, and the culture was then exposed to [14C]-sulfadoxine (7.5 MBq/litre (0.2 mCi/litre) prepared from a 1 mol/litre [14C]-sulfadoxine solution with a radioactivity of $37 \times 10^3$ MBq/litre) in WM or RP in a candle jar for 24 h at 37 °C. The culture medium was removed and the erythrocytes were washed three times in cold phosphate-buffered saline at pH 7.2. The packed cells were then resuspended with an equal volume of phosphate-buffered saline to make a 50% cell suspension. A 25-μl aliquot was placed on a fibre-glass filter, dried at 37 °C overnight, then dropped in dioxane scintillation cocktail and counted on a Beckman LS liquid scintillation counter. The number of erythrocytes per ml was estimated from a further 25-μl portion of cell suspension in a Neubauer chamber. Normal non-infected cells were cultured in either RP or WM and tested in an identical manner.

Statistical analysis

All parasite multiplication rates were tested by 2-way analysis of variance for the effects of drug concentration and culture medium. Incorporation of [14C]-sulfadoxine into infected and non-infected erythrocytes cultured in RP or WM was analysed in a single classification ANOVA (10).

RESULTS

The sensitivity of FCM2 and FCM5 to sulfadoxine at all concentrations was much higher in Waymouth culture medium than in RP (Fig. 1). There was no growth of the parasite population of either strain in WM medium containing only 0.5 mmol of sulfadoxine per litre, whereas, in RP, the parasite growth at this concentration was similar to that of the control group. A sulfadoxine concentration of 6.4 mmol/litre was required for 50% inhibition of FCM5 in RP.

Waymouth medium (without human serum) was found not to contain PABA, but 0.025 mg of PABA per litre was detected in complete WM containing 100 ml of human serum per litre. The concentration of PABA in RP was found to be 1.025 mg/litre, i.e., 41 times higher than that in WM. A further experiment, shown in Fig. 2, demonstrated that the FCM5 strain was inhibited in WM by 0.2 mmol/litre of sulfadoxine, but was unaffected in RP medium at this concentration (see Table 1, $P < 0.001$). The mean parasite multiplication rate in WM was only 0.48, a value achieved in RP only at a drug concentration of 6.4 mmol/litre. Thus, 32 times more sulfadoxine was required for 50% inhibition in the presence of a 41-fold higher concentration of PABA. Furthermore, the addition of 1 mg of PABA per litre of WM decreased the drug response of the parasite to the level seen in RP (Fig. 2).

The rate of incorporation of sulfadoxine in P. falciparum culture is shown in Table 2. The amount of drug incorporated into the non-infected erythrocytes was 15.9 times higher in WM (11 003 cpm) than in RP (692 cpm). The difference was even greater in parasite cultures in which 10% of

\[ \text{Sulfadoxine (mmol/litre)} \]

\[ \text{Parasite multiplication rate} \]

Fig. 1. Response of P. falciparum strains FCM2 and FCM5 to sulfadoxine in the Petri-dish test, showing mean parasite multiplication rate and standard deviation. \( \Delta \), FCM2 in RP; \( \Delta \), FCM2 in WM; \( \bigcirc \), FCM5 in RP; \( \bigcirc \), FCM5 in WM.

\( ^{c} \) Hoffmann-La Roche, Basel, Switzerland.
the erythrocytes were infected with trophozoites of *P. falciparum*; the counts were 62,900 cpm in WM and 2800 in RP (a 22.5-fold difference).

**DISCUSSION**

Our experimental results indicate that the apparent *in vitro* susceptibility of *P. falciparum* to sulfadoxine is influenced by the culture medium. RPMI was used in the first successful continuous culture of *P. falciparum* by Trager & Jensen (1) and has since been adopted internationally. Because of its PABA concentration of 1 mg per litre, this synthetic medium supports growth of malaria parasites *in vitro* (11). However, the PABA concentration in RPMI is much higher than the actual requirement and thus the medium is unsuitable for the *in vitro* investigation of antimalarial drugs that act by inhibiting either the utilization of PABA or the biosynthesis of folate precursors of the parasites. In 1950, Thurston (12) showed that PABA antagonizes the action of sulfadiazine against *P. berghei* in mice. The inhibition of *P. berghei* by a milk diet was demonstrated to be due to a PABA deficiency (13). The addition of human serum to the Waymouth formula medium provides sufficient PABA (250–300 μg/litre of serum) for the growth of the parasite. Studies in our laboratory (unpublished observations) have demonstrated that Waymouth medium supplemented with human serum is as good as RPMI in supporting growth and asexual multiplication of *P. falciparum*, as evidenced by glucose utilization, incorporation of [14C]-isoleucine, and the parasite multiplication rate. The observed sensitivity to sulfadoxine is thus not an artefact of an inappropriate culture medium. The kinetics of sulfadoxine incorporation into infected cells is being studied in our laboratory.

The test system reported here is being applied to the *in vitro* microtechnique (14) to assess sulfadoxine sensitivity using blood from patients in various endemic areas. It is hoped to obtain information on the sensitivity of *P. falciparum* in Thailand to sulfa drugs and to determine their minimum inhibitory concentrations. The *in vitro* cultivation of parasites using Waymouth formula medium will also provide a system for investigation of the pathway of dihydrofolate biosynthesis in malaria parasites.

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PLASMODIUM FALCIPARUM EN CULTURE CONTINUE: UN NOUVEAU MILIEU POUR L’ÉPREUVE DE SENSIBILITÉ À LA SULFADOXINE IN VITRO

Pour déterminer la réponse à la sulfadoxine de Plasmodium falciparum de Thaïlande, on a utilisé un système in vitro et la méthode employant des boîtes de Petri et une cloche à bougie. Les souches thaïlandaises FCM2 et FCM5 de P. falciparum ont été cultivées en milieux RPMI 1640 et Waymouth contenant 25 mmol de HEPES et 2 g de NaHCO₃ par litre, additionnés de 100 ml de sérum humain par litre (milieux désignés respectivement comme RP et WM). Le dosage de l’acide amino-4 benzoïque (PABA) dans les deux milieux a révélé des concentrations de 1,025 mg/litre dans RP et de 0,025 mg/litre dans WM. Les réponses des deux isolements de P. falciparum à la sulfadoxine ont été très faibles dans RP, où il fallait une concentration de médicament de 6,4 mmol/litre pour obtenir une inhibition de 50% de la croissance des parasites, alors qu’il n’y avait plus aucune croissance dans le milieu WM à une concentration de 0,5 mmol de sulfadoxine par litre. D’autres expériences ont confirmé une inhibition hautement significative dans le milieu WM par une concentration du médicament aussi faible que 0,2 mmol/litre.

La différence entre les résultats observés dans les milieux RP et WM a été attribuée à une compétition entre le PABA et la sulfadoxine, analogue du PABA. Cela a été confirmé par le fait que 1) la croissance des parasites n’était que faiblement modifiée par la sulfadoxine en milieu WM auquel du PABA supplémentaire avait été ajouté, et que 2) la sulfadoxine marquée par ¹⁴C était incorporée dans les érythrocytes beaucoup plus rapidement en milieu WM qu’en milieu RP.

REFERENCES