TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE, VOL. 75, NO. 2, 1981

Resistance of ten Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests

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Summary

In vitro drug resistance tests of ten isolates of *Plasmodium falciparum* from three different collection points in Central Thailand have been carried out, and the results compared with those of similar tests with a drug-sensitive West African isolate. Judged by concentration of drug tolerated, the Thai isolates appeared to be about 10 times as resistant to chloroquine, and usually about 10^5 times as resistant to pyrimethamine, as the African isolate. A little variation amonst the Thai isolates was detected.

Introduction

Drug resistance is of particular practical importance in *Plasmodium falciparum*: we have, therefore, included in our study of genetic variation amongst Thai isolates of these parasites some tests for resistance to two widely used drugs—chloroquine and pyrimethamine. The development of an *in vitro* culture method (TRAGER & JENSEN, 1976) has made it possible to measure drug resistance under controlled laboratory conditions (NGUYEN-DINH & TRAGER, 1978; RICHARDS & MAPLES, 1979). The short term *in vitro* method of RIECKMANN *et al.* (1968), and especially the more recent micro-version (RIECKMANN *et al.*, 1978) are very valuable for rapid field tests with small quantities of parasitized blood, but are limited by the fact that the action of the drug is measured during only a part of the parasite's life-cycle (maturation of schizonts from ring forms over a 24-hour period), and also by the fact that the tests can be carried out once only for each isolate, after which the sample is lost. Hence deviating results cannot be checked by this method, and isolates cannot be subsequently studied for other characteristics (e.g. for enzyme variants).

In the work to be described here a method similar to those of NGUYEN-DINH & TRAGER (1978) and RICHARDS & MAPLES (1979) is used, with some modifications. The isolates studied were maintained for long periods in culture and it was possible to check that the parasites were growing regularly before and during the tests. Moreover, tests of a given isolate could be repeated as often as necessary, until consistent results were obtained.

Material and Methods

Nine isolates of *P. falciparum* from three different endemic areas in Central Thailand were selected for study, and one isolated from West Africa (The Gambia) as a drug-sensitive standard. Usually the isolates were maintained in culture for at least 10 cycles (20 days) before study of drug resistance. (In two cases, however, a shorter period was considered sufficient since parasite morphology was seen to be satisfactory). By the time of the drug-tests, growth of most cultures was unsynchronized. The culture technique used was that described by TRAGER & JENSEN (1976) (candle-jar method). Group AB blood and serum was used. Before using any samples of red blood cells or serum for growing parasites during the drug test, careful checks were made to confirm that parasite growth was satisfactory.

As a further check on the culture conditions during the tests, as well as the reproducibility of the effect of a given drug concentration, one isolate (K1), was used as a standard for comparison with all the others, i.e. tests for each isolate were compared with tests for K1, run simultaneously. In some tests, the drug-sensitive African isolate (G1) was included as an additional control. If growth of the K1 and G1 standards, or of any of the tested isolates placed in normal drug-free medium, was less than optimal, or if the response of the "standard" isolates to the drugs deviated from that normally found, the results were discarded.

The tests were carried out as follows. 100μl of complete RPMI 1640 medium with 10% serum (without drug), were pipetted into each of four wells of a microtitre plate, and the same medium containing a graded series of five drug concentrations was pipetted into a series of 20 wells, (four wells for each drug concentration). 10 μl of packed parasitized cells from the culture to be tested were added and stirred briefly to mix cells and medium.

The parasitaemia at the start of the experiment was never above 1% nor below 0·3%, and after 48 hours there was usually about a four-fold increase in parasitaemia in the undrugged series. The microtitre plate was placed in a candle-jar and incubated at 36 to 37°C. After 24 and 48 hours the medium was withdrawn and replaced with fresh drug-free or drug-containing medium. Thin blood films were made after 48 and 72 hours and stained with Giemsa. Each test was carried out in a different well, so that blood samples could be withdrawn from some wells without disturbing cultures to be grown longer.

The drug preparations were made up as follows:
(1) *Chloroquine*—Chloroquine diphosphate (Resochin, Bayer) was dissolved in complete RPMI 1640 medium to a concentration of 20 μg per ml., and added to the complete RPMI 1640 medium with 10% serum to give the finally desired concentration.

Table I—Resistance of Thai isolates of *P. falciparum* to chloroquine and pyrimethamine

Isolate No.	Place collected	Date collected	Chloroquine ug/ml 0·01	0·03 (= 10^{-7}M)	0·05	0·1	0·3 (= 10^{-6}M)	0·45	Date of Test	Pyrimethamine M 10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	$5-10^{-5}$	10^{-4}	Date of Test
K_1	Kanchanaburi	29.1.79		✓	✓	✓	✓ x	x	various			✓	✓	✓	✓	x		various
K_{28}		23.11.79		✓	✓	✓	✓ x	x	22.12.79			✓	✓	✓	✓	✓	x	17.12.79
K_{30}		30.11.79		✓	✓	✓	✓ x	x	22.12.79			✓	✓	✓	✓	x	x	17.12.79
S_2	Sriracha	27.11.78		✓	✓	✓	✓ x	x	11.1.79									
S_3		13.11.78		✓	✓	✓	✓ x	x	4. 1.79			✓	✓	✓	✓	x	x	30. 9.79
S_{118}		1.7.79		✓	✓	✓	✓ x	x	8. 5.79			✓	✓	✓	✓	x	x	12. 1.80
S_{151}		2.11.79		✓	✓	✓	✓ x	x	30.12.79									
PB_1	Praputhabath	7. 1.80		✓	✓	✓	✓	x	12.1.80			✓	✓	✓	✓	x	x	12. 1.80
PB_2		7. 1.80		✓	✓	✓	✓	✓	14.1.80			✓	✓	✓	✓	✓	x	14. 1.80
PB_{14}		2.10.80		✓	✓	✓	✓x	x	7. 4.80	✓	✓	✓	x	x	x	x	x	11.11.80
G_1	Gambia		✓	x	x	x	x	x	1. 3.79	✓	x	x	x					1. 3.79
									8. 8.79									8. 8.79

Explanation
✓ growth uninhibited by drug
✓ x growth sometimes inhibited sometimes uninhibited or growth at reduced rate
x no growth after 48 hrs and 72 hrs.

(ii) *Pyrimethamine*—(Wellcome) was dissolved initially in 0·5% lactic acid to a concentration of 10^{-3}M of drug, as described by RICHARDS & MAPLES (1979).

Culture media and drug solutions were sterilized by passage through millipore filters. Drug solutions were stored at 4°C throughout the study.

Results

The results are given in Table I. We have not attempted to express them numerically, since in our experience wide fluctuations occur in the estimated numbers of parasites at different stages recorded by counts based on number of parasite-containing red cells seen in a given number of microscope fields. We consider it more satisfactory to record simply whether or not growth has occurred normally or is inhibited after a certain period in the presence of drug. This method does not of course permit us to establish the significance of any variations between isolates which may appear.

Inspection of Table I will show that the Thai isolates examined here do not show much variation amongst themselves in resistance to either chloroquine or pyrimethamine. Some minor variations may occur, e.g. isolate PB2 grew apparently normally in chloroquine at a concentration of 0·4μg/ml, which inhibited growth of all the other isolates. Two isolates—K28 and PB2—grew in pyrimethamine at a concentration of 5×10^{-5}M which inhibited all the other isolates. One isolate—PB14—was more sensitive to pyrimethamine than any other tested, although it was more resistant than the Gambian isolate (G1).

All the Thai isolates were more resistant than the African one (G1), to both drugs. With regard to chloroquine there was approximately a ten-fold difference in the concentration of drug which could be tolerated by Thai and African isolates, and with regard to pyrimethamine the difference was about 10^5 times (though it is not of course implied that other African strains are necessarily as sensitive as the one used here).

Discussion

In general, the degree of resistance found was within the range reported by other workers for comparable parasite isolates. NGUYEN-DINH and TRAGER (1978) found that a highly resistant (R III) isolate from Viet Nam was completely inhibited by 0·3μg/ml chloroquine ($M \times 10^{-6}$), and a sensitive Gambian isolate by 0·1μg/ml chloroquine. As regards pyrimethamine, RICHARDS & MAPLES (1979) found that a Nigerian isolate was eliminated by a concentration of 10^{-8}M, but only partly inhibited by 10^{-9}M. It may be concluded that this Nigerian isolate was somewhat more resistant to pyrimethamine than the Gambian isolate (G1) tested here. Exact comparisons are not, however, possible since the methods used by the cited authors were not precisely the same as ours. Comparison with the results of RIECKMANN *et al.* (1978) using the short term micro-test are more difficult, in view of the different conditions and different measurements made.

It is noteworthy that most of the Thai isolates here examined tolerate an extremely high concentration of pyrimethamine ($M \times 10^{-5}$). If most other Thai isolates are equally resistant, the widespread use of pyrimethamine as a "presumptive" treatment would seem to have little or no value.

Although these results indicate that the amount of variation in drug resistance within the area sampled is slight, it is not excluded that further studies with more isolates from the same area, or from other endemic areas might reveal greater variation. As will be seen from our parallel study on enzyme variation (THAITHONG *et al.*, 1981), some genetic variants occur at a very low frequency.

Nevertheless the present, provisional, conclusion is that the Thai strains are rather uniformly resistant both to chloroquine and pyrimethamine. The high degree of resistance to the latter drug, and the widespread use of Fansidar (sulphadoxine and pyrimethamine), indicate the need to carry out a similar study on resistance to sulpha-drugs, both alone and in combination with pyrimethamine.

Acknowledgement

This investigation received support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and from the Wellcome Trust.

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Accepted for publication 12th April, 1980.