In vitro sensitivity of southern African reference isolates of Plasmodium falciparum to chloroquine and pyrimethamine

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The in vitro sensitivity to chloroquine and pyrimethamine of 19 culture-adapted southern African reference isolates of Plasmodium falciparum was determined using a 48-hour assay. Four isolates collected in KwaZulu, Natal, were sensitive to chloroquine, and one of these was sensitive to the drug in vivo. Eight isolates from KwaZulu or Mozambique were resistant to chloroquine in vitro. Six of these isolates were chloroquine-resistant in varying degrees in vivo. Four of five isolates from north-eastern Transvaal and two clinically chloroquine-resistant Malawian isolates were resistant to chloroquine in vitro. A wide range of pyrimethamine susceptibilities was detected (0.01 μmol/l to >3.0 μmol/l), although most isolates were inhibited at 0.1 μmol/l, indicating a low level of resistance. These results confirm the presence of both chloroquine and pyrimethamine resistance in the endemic areas of South Africa. This has serious implications for the prophylaxis and treatment of P. falciparum malaria in South Africa.

Introduction

One of the major problems facing malaria control is the resistance that Plasmodium falciparum develops to antimalarial drugs. Until the late 1970s, chloroquine was used very successfully in Africa but, since the first reports of chloroquine resistance in the continent (1, 2), in vivo and in vitro resistance to the drug has been documented in many African countries, including Mozambique, Angola, Namibia (3), Zimbabwe (4) and Swaziland (5).

Chloroquine resistance was first noted in South Africa in 1985. Many malariorologists outside the country are unaware of the existence of chloroquine resistance in South Africa, although attention was drawn to it at the time (6–8). Only minimal laboratory data on chloroquine sensitivity have previously been published for malarial isolates from South Africa. Resistance of P. falciparum to pyrimethamine is also widespread in Africa (9), although it has not been monitored as closely as chloroquine resistance.

The proportion of cases of P. falciparum malaria from northern KwaZulu (an endemic area in the province of Natal, South Africa) that failed to respond to treatment with the chloroquine–pyrimethamine combination rose from 0 in 1983 to 16.1% and 21.2% in field and hospital cases, respectively, in 1987 (10). The in vitro chloroquine sensitivity of isolates from KwaZulu has previously been investigated to a limited extent, but no studies of pyrimethamine sensitivity have hitherto been carried out in South Africa.

As part of a characterization study of southern African isolates, we determined the in vitro sensitivity to chloroquine and pyrimethamine of 19 culture-adapted reference strains of P. falciparum. Such data are particularly useful because these isolates have been well-characterized genetically (11, 12). Some correlation has been found between the in vitro data and the in vivo sensitivity to chloroquine or a combination of chloroquine and pyrimethamine, and these findings are also reported.

Materials and methods

Plasmodium falciparum isolates

Since 1984 we have successfully maintained the culture-adapted Gambian FCR-3 strain of P. falciparum (13) in the laboratory. In addition, 19 southern African isolates of P. falciparum were obtained from patients who had contracted malaria between July 1984 and February 1987. The isolates were maintained cryopreserved and/or as cultured parasites and could, therefore, be used as reference material; the geographical origins of infection and dates of collection of these reference isolates are shown in Table 1. RSA 1–12 are listed in Table 1 as KwaZulu isolates (but see Discussion). RSA 2 was from a patient who subsequently responded to treatment with 10 tablets of Daraclor (1 tablet = 150 mg
chloroquine base + 15 mg pyrimethamine base). RSA 3, 4, and 5 were from persons who reported to a clinic, but for whom no clinical data were available. RSA 6 was from a patient who failed to respond to chloroquine treatment. RSA 1, 7, 8, 9, 10, 11, and 12 were from apparently healthy labourers who were employed on farms in a nonmalarious area of Natal. All of these Natal cases are thought to have originated in KwaZulu. The individuals from whom RSA 8, 10 and 11 were collected all failed to respond to treatment with four tablets of Daraclor, but were cleared of parasites when given a further 10 tablets. Those from whom RSA 7 and RSA 12 were obtained did not respond to treatment with 10 tablets of Daraclor. Clinical data on the patients from whom RSA 1 and RSA 9 were collected were unavailable. Isolates RSA 13–17 were from patients from the endemic area of north-eastern Transvaal. Three of these isolates (RSA 15–17) were from persons who had been cleared of symptoms after receiving Daraclor. However, no information about the in vivo response of the parasites to the chemotherapy is available, since no blood smears were taken following the treatment. Clinical data on the remaining two patients from north-eastern Transvaal are not available. Isolates MW 1 and MW 2 were from patients who became infected while visiting Malawi; in both cases the parasites were resistant to chloroquine treatment.

The isolates were established and maintained in suspension culture as described previously (14).

Briefly, parasites were grown in a 5% suspension of O-positive red blood cells in RPMI 1640 culture medium, prepared as described by Jensen & Trager (15), and supplemented with glucose (4 g/l), hypoxanthine (44 mg/l), gentamicin (50 mg/l), and 10% human AB serum. The cultures were grown in tissue culture flasks, which were filled with a mixture of 3% oxygen, 4% carbon dioxide and 93% nitrogen, and incubated at 38°C on a shaker platform. The medium was changed daily, and infected erythrocytes were diluted with washed, uninfected red blood cells every 3 or 4 days, depending on the growth of the cultures. Once adapted to the culture conditions, the isolates were tested for in vitro drug sensitivity.

**In vitro test**

The in vitro sensitivities of the culture-adapted isolates to chloroquine and pyrimethamine were determined using the 48-hour test described by Nguyen-Dinh & Payne (16), which was modified as described below.

Infected red blood cells were diluted with fresh, washed O-positive erythrocytes to a parasitaemia of 0.1–0.9%, and a 2% suspension in the culture medium was prepared. Aliquots (0.5 ml) of this suspension were added to the wells of 24-well plates that had been preseeded with chloroquine and pyrimethamine to give final concentrations of 0, 0.03, 0.06, 0.1, 0.3, 0.6, and 1.0 μmol/l of chloroquine and 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 μmol/l of pyrimethamine. Each isolate was tested in three wells
at each drug concentration. The plates were agitated for a few seconds to dissolve the drugs and were then placed in a desiccator through which the above-described gaseous mixture of oxygen, carbon dioxide, and nitrogen was passed for approximately 3 minutes. The desiccator was then tightly sealed and placed in an incubator for 48 hours at 38°C. After 24 hours the plates were agitated to resuspend the settled red blood cells and the gaseous mixture was again passed through the desiccator. Parasites were counted microscopically from Giemsa-stained thin films prepared at the beginning and the end of the test. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration at which no parasite growth took place. Each isolate was tested on three different occasions between August 1986 and September 1987. The period between initiation of a culture and the initial in vitro test ranged from 1 to 29 months. Subsequent tests were carried out within 12 months of the initial test.

Results

Initial tests

The results of the initial in vitro tests are presented in Table 1. Four KwaZulu isolates (RSA 2, 3, 4 and 5) were inhibited at chloroquine concentrations of 0.1 μmol/l or less, indicating sensitivity to the drug (17). All the other KwaZulu isolates (i.e., RSA 1, 6, 7, 8, 9, 10, 11, and 12) were chloroquine-resistant in vitro, having MICs of 0.3 or 0.6 μmol/l. Only one of the isolates from north-eastern Transvaal (RSA 13) was sensitive to chloroquine, having been inhibited at a concentration of 0.06 μmol/l. The remaining four isolates from this region were resistant in vitro, since they had MICs of ≥0.3 μmol/l. Both clinically chloroquine-resistant Malawian isolates were also resistant in vitro. The Gambian FCR-3 strain was inhibited at 0.6 μmol/l chloroquine.

In the initial tests, the pyrimethamine MICs for the southern African isolates ranged from 0.01 μmol/l to >3.0 μmol/l, with the majority of the strains tested having been inhibited at 0.1 μmol/l. Three isolates were inhibited at ≤0.03 μmol/l, while three were not inhibited at the highest concentration tested (3 μmol/l).

Change in drug sensitivity over time

Table 2 shows the results of the three chloroquine and pyrimethamine in vitro tests. The chloroquine MIC for most strains fluctuated between two values during the test period, but was constant for RSA 2, 4 and 11, as well as for FCR-3. Variation in the results of the pyrimethamine test was infrequent and only six cultured isolates (RSA 1, 5, 7, 10 and 13, and MW 2) yielded different pyrimethamine MICs at different times. The MW 2 isolate was not inhibited at a drug concentration of 3.0 μmol/l in the initial test but in subsequent tests was inhibited at a drug concentration of 0.1 μmol/l.

Discussion

In vivo/in vitro relationships

All southern African isolates tested were inhibited at in vitro chloroquine concentrations of ≤0.6 μmol/l except for one isolate from the north-eastern Transvaal, which was inhibited at 1.0 μmol/l in one of the tests. These MICs are comparable with those of other African isolates (18), but are lower than those of the Thai isolates investigated by Thaithong et al. (19), which lay in the range 0.1–1.5 μmol/l, with the majority being 1.0 μmol/l or 1.5 μmol/l.

Isolate RSA 2 (which was sensitive to chloroquine and pyrimethamine in vitro) and isolates RSA 3, 4 and 5 were all sensitive to chloroquine in vitro. These isolates were collected early in 1985 from patients who reported to a hospital or a clinic in the endemic malarial area of KwaZulu, which borders Mozambique. The first cases of in vitro chloroquine resistance from this area were discovered in 1985 (7). In contrast, field studies in KwaZulu in May 1987 and January 1988, after chloroquine had been used extensively in the area for prophylaxis and treatment, revealed widespread in vitro chloroquine

Table 2: Response of southern African reference isolates of Plasmodium falciparum to chloroquine and pyrimethamine in the 48-hour in vitro test (results of 3 tests)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSA 1</td>
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<td>0.3</td>
<td>0.3</td>
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<tr>
<td>RSA 2</td>
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<td>0.06</td>
<td>0.06</td>
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<tr>
<td>RSA 3</td>
<td>0.06</td>
<td>0.06</td>
<td>0.1</td>
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<tr>
<td>RSA 4</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>RSA 5</td>
<td>0.1</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>RSA 6</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
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<tr>
<td>RSA 7</td>
<td>0.6</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>RSA 8</td>
<td>0.6</td>
<td>0.3</td>
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<tr>
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<td>RSA 10</td>
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<tr>
<td>RSA 11</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
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<tr>
<td>RSA 12</td>
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<td>0.3</td>
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<tr>
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<td>0.06</td>
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<td>0.3</td>
<td>0.3</td>
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<tr>
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<td>0.3</td>
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<td>RSA 17</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>MW 1</td>
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<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>MW 2</td>
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<tr>
<td>FCR-3</td>
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<td>0.6</td>
</tr>
</tbody>
</table>

resistance (20). These resistant parasites were probably introduced into the local *Plasmodium falciparum* population by movement of people across the border from Mozambique. In 1986 the problems associated with the influx of Mozambicans were highlighted when it became apparent that a large proportion of malaria cases detected in Natal had been imported from Mozambique (21). Six isolates included in the present study (RSA 7, 8, 9, 10, 11 and 12) were obtained from labourers employed in 1986 on sugar farms in this nonendemic area. Isolate RSA 1 was from a labourer who worked in the same area in 1985. Although these infections were thought to have originated in KwaZulu, they may have been imported from Mozambique. Refugees are often reluctant to admit that they are Mozambican and prefer to state that they are residents of KwaZulu. The results of the present study indicate that four of five isolates from north-eastern Transvaal, which is also adjacent to Mozambique, were resistant to chloroquine in vitro. These resistant strains were probably imported into this endemic region. As expected from the clinical histories of their hosts, the Malawian isolates were chloroquine-resistant in vitro.

A wide range of pyrimethamine sensivities (0.01 to >3.0 μmol/l) was exhibited by the southern African isolates, as in Thailand, where the in vitro sensitivities were 0.001–100.0 μmol/l (19). Since pyrimethamine was used in combination with chloroquine, it was not possible to determine the in vivo response of the southern African isolates to pyrimethamine alone. Most of the isolates were inhibited in vitro at 0.1 μmol/l, which suggests that the strains are not fully susceptible to pyrimethamine. However, the relationship between the in vivo response to pyrimethamine and the in vitro MIC has not yet been clearly defined. Based on a correlation between the in vivo and in vitro responses of *P. falciparum* isolates from 10 countries, Smalley & Brown concluded that isolates inhibited in vitro at 0.04 μmol/l pyrimethamine are likely to be sensitive to the drug, whereas those which grow in concentrations of >0.1 μmol/l are likely to be resistant (22). However, Spencer et al. have reported that one clinically pyrimethamine-sensitive Kenyan isolate had an MIC of 0.1 μmol/l in a 48-hour in vitro test, while three other isolates that were sensitive to the drug were inhibited at concentrations of ≤0.03 μmol/l (23). Clearly further studies of the in vivo and in vitro sensitivity to pyrimethamine of *P. falciparum* isolates are required before this controversy can be resolved.

**Stability of drug sensitivity in cultured isolates**

A chloroquine concentration of 0.6 μmol/l of chloroquine was required for the in vitro inhibition of the FCR-3 strain. This isolate, which was initially cul-
24-hour pyrimethamine in vitro test indicated that those *Plasmodium falciparum* isolates inhibited at 20.0 μmol/l were sensitive to Fansidar in vivo, while six of seven isolates that grew in pyrimethamine levels of ≥ 20.0 μmol/l were resistant to the drug combination in vivo. The southern African isolates were not tested at pyrimethamine levels of > 3.0 μmol/l but only three isolates were not inhibited at 3.0 μmol/l, and the majority of the MICs were considerably less than this concentration. We would therefore not expect resistance to Fansidar to be a problem in South Africa, at least not in the near future.

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**Résumé**

**Sensibilité in vitro à la chloroquine et à la pyriméthamine d’isolats de référence de* Plasmodium falciparum *provenant d’Afrique du Sud**

En Afrique du Sud, *Plasmodium falciparum* présente une certaine résistance à la fois à la chloroquine et à la pyriméthamine, médicaments couramment utilisés dans le pays pour la prophylaxie et le traitement du paludisme. La sensibilité in vitro à la chloroquine et à la pyriméthamine de 19 isolats de référence, adaptés à la culture, de *P. falciparum* bien caractérisés du point de vue génétique a été déterminée au moyen d’une épreuve de 48 heures. Les données obtenues servent de données de référence pour les études ultérieures de pharmacosensibilité.

Quatre isolats recueillis au KwaZulu étaient sensibles à la chloroquine; l’un d’entre eux était également sensible au médicament in vivo. Huit autres isolats provenant du KwaZulu ou du Mozambique étaient résistants à la chloroquine in vitro. Six d’entre eux présentaient divers degrés de résistance à la chloroquine in vivo. Quatre des cinq isolats provenant du nord-est du Transvaal et deux isolats provenant du Malawi et pour lesquels une résistance clinique à la chloroquine était observée, étaient résistants à la chloroquine in vitro. Des degrés très divers de sensibilité à la pyriméthamine ont été observés (0,01 μmol/l à > 3,0 μmol/l), bien que la plupart des isolats aient été inhibés à la concentration de 0,1 μmol/l, ce qui traduit une résistance faible.

La sensibilité des isolats d’Afrique du Sud à la chloroquine et, dans une moindre mesure, à la pyriméthamine, a été variable au cours de la période étudiée. Cela a été probablement dû à la présence de clones de pharmacosensibilité différente qui, en raison d’une croissance asynchrone, se trouvaient présents dans la culture dans des proportions différentes aux divers moments.

Nos résultats confirment la présence d’une résistance, tant à la chloroquine qu’à la pyriméthamine, dans les zones d’Afrique du Sud où le paludisme est endémique; ces observations ont des conséquences graves pour la prophylaxie et le traitement du paludisme à *P. falciparum*. Actuellement, l’association chloroquine-pyriméthamine est couramment recommandée en Afrique du Sud à la fois pour la prophylaxie et le traitement du paludisme, bien que l’intérêt de cette association médicamenteuse ait été récemment mis en doute. L’emploi continu de ces médicaments risque en effet d’entraîner une prévalence accrue du paludisme pharamcorésistant et peut également donner lieu à une plus forte résistance stable aux deux médicaments. En Afrique du Sud, une résistance étendue à la pyriméthamine pourrait menacer l’efficacité des autres associations concernant ce médicament, comme la pyriméthamine-sulfadoxine.

La résistance à la pyriméthamine pourrait être un bon indicateur de la résistance au Fansidar (pyriméthamine + sulfadoxine). Des recherches effectuées en Papouasie-Nouvelle-Guinée ont montré que les isolats inhibés à 20 μmol/l de pyriméthamine lors d’une épreuve in vitro de 24 heures étaient sensibles au Fansidar in vivo, tandis que la plupart des isolats qui poussaient encore à ≥ 20 μmol/l étaient résistants à l’association in vivo. La plupart des isolats sud-africains étaient inhibés à des taux de pyriméthamine bien inférieurs à 3 μmol/l, concentration la plus élevée utilisée lors de cette étude, seuls trois isolats poussant encore en présence de 3 μmol/l de pyriméthamine. Nous ne pensons donc pas que la résistance au Fansidar puisse poser un problème en Afrique du Sud, au moins dans un proche avenir.

**References**

J. A. Freese et al.


