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IN VIVO AND *IN VITRO Plasmodium falciparum* RESISTANCE TO CHLOROQUINE, AMODIAQUINE AND QUININE IN THE BRAZILIAN AMAZON

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SUMMARY

In order to study the chemoresistance of *Plasmodium falciparum* to commonly used antimalarial drugs in Brazil the authors have studied ten patients with falciparum malaria, acquired in the Brazilian Amazon region. Patients were submitted to *in vivo* study of drug sensitivity, after chemotherapy with either 4-aminoquinolines (chloroquine or amodiaquine) or quinine. Adequate drug absorption was confirmed by standard urine excretion tests for antimalarials. Eight patients could be followed up to 28 days. Among these *in vivo* resistance (R I and R II responses) was seen in all patients who received 4-amino-quinolines. One patient treated with quinine exhibited a R III response. Peripheral blood samples of the same patients were submitted to *in vitro* microtests for sensitivity to antimalarials. Out of nine successful tests, resistance to chloroquine and amodiaquine was found in 100% and resistance to quinine in 11.11% of isolates. Probit analysis of log dose-response was used to determine effective concentrations EC_{50}, EC_{90} and EC_{99} to the studied drugs. Good correlation between *in vivo* and *in vitro* results was seen in six patients. The results emphasize high levels of *P. falciparum* resistance to 4-aminoquinolines and suggest an increase in resistance to quinine in the Brazilian Amazon region, reinforcing the need for continuous monitoring of drug sensitivity to adequate chemotherapy according to the most efficacious drug regimens.

KEYWORDS: Malaria; *Plasmodium falciparum*; Chemotherapy; Resistance.

INTRODUCTION

Antimalarial chemotherapy is one of the cornerstones in the control of malaria transmission in different parts of the world [9, 22, 32]. Moreover the adequate use of antimalarial drugs is essential as a means of reducing the high morbidity and mortality associated to falciparum malaria in non-immune hosts. Therefore knowledge of the geographical distribution of *Plasmodium falciparum* resistant strains and the identification of the severity of resistance are important in the choice for efficacious therapeutic regimens. Failures in the treatment of falciparum malaria with quinine in Brazil were originally reported in the beginning of this century [11]. However the precise distinction between treatment failure and parasite chemoresistance could not be assessed then. For this to be achieved *in vivo* or *in vitro* sensitivity studies are required. It is also important to have the results of both tests correlated. so as to determine whether *in vitro* resistance may be reliably taken as a predictor of treatment outcome. The authors have therefore carried out a parallel *in vivo* and *in vitro* study of *Plasmodium falciparum* sensitivity to chloroquine, amodiaquine and quinine in patients with falciparum malaria who acquired the disease in the Brazilian Amazon region.

PATIENTS AND METHODS

From August 1986 to August 1990, ten patients with falciparum malaria, acquired in the Brazilian Amazon region, were enrolled to the present study. They were diagnosed at the Malaria Laboratory-SUCEN or at the Hospital das Clinicas, School of Medicine, University of São Paulo in São Paulo, Brazil (far from endemic areas of malaria transmission). Their finger punctures yielded asexual parasitemias between 2,000 and 10,000 ring forms per mm^3. Patients with primary infection and those with clinical evidence of severe infection (cerebral or lung involvement, renal failure or jaundice) were excluded from the study. Likewise patients referring treatment with 4-aminoquinolines (chloroquine or amodiaquine) or quinine up to 15 days prior to admission to the study, those treated with sulphonamides in the 30 days before admission and those with positive standard urinary excretion tests for antimalarials on admission were also excluded from the study.

IN VIVO STUDY

For the *in vivo* study of *Plasmodium falciparum* sensitivity to antimalarials, patients were admitted to the Infectious Diseases Ward of the Hospital das Clínicas, School of Medicine, Univer-

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sity of São Paulo for seven days. Chemotherapy was given at random, according to the following regimens:

- Group A (4-aminoquinolines): Chloroquine diphosphate or amodiaquine hydrochloride, 600 mg orally at first, followed by 300 mg after 6, 24 and 48 hours (total oral dose = 1500 mg);

- Group B (quinine): Quinine sulphate, 500 mg orally at three-hour intervals for 7 days.

Antimalarials were given under supervision. During the first seven days after having started treatment patients were daily evaluated, so as to have fever clearance and disappearance of clinical symptoms verified. They were submitted to dailly finger punctures for parasitemia assessment. Drug absorption was tested by standard urinary excretion tests for antimalarials using Dill-Glazko [20] and Mayer-Tanret [33] reagents on the second day after starting chemotherapy. On the seventh day patients were discharged from hospital and instructed to come back for weekly follow-up visits on the 14 th, 21st and 28th days after treatment had started. *In vivo* response to chemotherapy was evaluated according to criteria standardized by the World Health Organization [34].

IN VITRO STUDY

Blood samples were collected from ten patients admitted to the *in vivo* study and submitted to an *in vitro* microtest of *Plasmodium falciparum* sensitivity to chloroquine, amodiaquine and quinine, according to a modified technique, following the description by RIECKMANN et al[25]. Stock drugs (chloroquine diphosphate, amodiaquine hydrochloride and quinine sulphate at 1 mMol/L concentrations), used for the *in vitro* study, were provided by the World Health Organization. Drug dillutions were prepared before each microtest. Cut-offs for determining drug sensitivity were adopted as recommended in literature: chloroquine = 1.2 microMol/L; amodiaquine = 0.4 microMol/L and quinine = 51.2 microMol/L [13, 18,27].

Probit analysis [14] was used to estimate the percentual inhibition of schizont development in relation to the decimal logarithm of antimalarial concentrations. Therefore EC_{50}, EC_{90} and EC_{99} - drug concentrations capable of inhibiting 50, 90 and 99% of parasite development were calculated. Linear regression curves of these parameters were obtained for each of the studied drugs (data not shown).

RESULTS

Ten patients with falciparum malaria, diagnosed by thick blood films, were included in the study. Nine patients were male and one female. Their ages ranged from 21 to 56 (mean 21, median 25.5). All patients had acquired malaria in the Brazilian Amazon region (four in the Sate of Rondônia, four in Mato Grosso and two in Pará). Their initial parasite counts ranged from 2,040 to 80,400/mm^3 (geometric mean 21,595/mm^3). Eight patients could be followed up to 28 days in the *in vivo* study and in nine patients successful *in vitro* parasite development was obtained using the microtest of sensitivity to chloroquine, amodiaquine and quinine.

IN VIVO STUDY

Group A consisted of three patients treated with chloroquine and two with amodiaquine. All five patients in Group B received quinine. Initial parasite counts were not significant different in both groups (Geometric means: 17,516 in Group A and 22,411 in Group B) as compared by the t-Student test ($p = 0.05$).

As two subjects refused being followed as in - patients for seven days, only eight patients were analysed for results of the *in vivo* study. Of these two had been treated with chloroquine, one with amodiaquine and five with quinine.

Standard urinary tests for antimalarials, using Dill-Glazko and Mayer-Tanret reagents were positive in all patients included in the *in vivo* study on the second day after chemotherapy had started.

Parasite counts in response to chemotherapy are depicted in Table 1.

TABLE 1

Asexual parasitemia from patients followed up in an *in vivo* study of *Plasmodium falciparum* sensitivity to chloroquine, amodiaquine and quinine and final *in vivo* therapeutic response according to W.H.O. classification.

Patient	Asexual parasitemia on day											Therapeutic response
	d0	d1	d2	d3	d4	d5	d6	d7	d14	d21	d28	
A-1	15360	31920	2160	360	120	neg	neg	neg	neg	-	-	R I
A-2	16380	8700	120	neg	neg	neg	neg	neg	neg	neg	460 80	R I
A-3	21380	19920	5520	300	8040	11400	14400	15000	-	-	-	R II
B-1	73800	8400	26200	5520	neg	neg	neg	neg	neg	neg	neg	S
B-2	2040	840	360	240	neg	neg	neg	neg	neg	neg	neg	S
B-3	57240	170640	91200	300000	-	-	-	-	-	-	-	R III
B-4	8160	1320	180	neg	neg	neg	neg	neg	neg	neg	neg	S
B-5	80400	20160	200000	60720	3960	neg	neg	neg	neg	neg	neg	S

d0 - day chemotherapy was started
d 1, 2, 3, ..., 28 - number of days afterwards
neg: negative asexual parasitemia
(-): parasite count worthless for the present study

The patient treated with amodiaquine in Group A had a parasitemia cleared on the third day, but developed a relapse on the 28th (R I response). One of the patients, that received chloroquine, had blood parasites cleared on the fifth day and relapsed on the 16th (R I response) and the other still had asexual parasitemia by the seventh day (R II response). Therefore *in vivo* resistance to 4 aminoquinolines was demonstrated in 100% of treated patients. Relapsed patients were subsequently treated with mefloquine 750 mg as a single oral dose, or quinine sulphate 500 mg at eight-hour intervals for three days plus tetracycline 250 mg at six-hour intervals for seven days and were cured.

In Group B four patients had their parasitemia cleared within the first week after chemotherapy had started and exhibited no relapses until the 28th day of follow-up (S response). Parasite clearance time was 3 days (in one patient), 4 days (in two), and 5 days (in one). In one patient increasing parasite counts were noticed in the first three days after chemotherapy and he showed signs of clinical worsening, with depressed consciousness. He was therefore regarded as exhibiting a R III response and was subsequently treated with intravenous quinine hydrochloride 500 mg at eight-hour intervals for seven days and a single oral dose of mefloquine (750 mg) and was cured.

Considering patients that were cured in the *in vivo* study, mean parasite clearance time was similar in Groups A and B (4 days).

IN VITRO STUDY

In the microtest for sensitivity to chloroquine, amodiaquine and quinine, successful *in vitro* schizont development could be obtained in nine blood samples. *In vitro* resistance to choroquine and amodiaquine was demonstrated in all samples and one isolate (11.11%) was resistant to quinine as well.

The probit analysis of log dose-response was employed to calculate effective concentrations of antimalarials, capable of inhibiting 50%, 90% and 99% of parasite development (EC_{50}, EC_{90} and EC_{99}). These parameters are shown in Table 2.

TABLE 2

Inhibitory concentrations of chloroquine, amodiaquine and quinine (micromol/Liter) of 50% (EC_{50}), 90% (EC_{90}) and 99% (EC_{99}) of *Plasmodium falciparum* schizont development in *in vitro* sensitivity microtests.

Drug	EC_{50}	EC_{90}	EC_{99}
chloroquine	5.17	148.69	2,297.72
amodiaquine	0.40	8.40	100.34
quinine	3.37	48.04	144.36

CORRELATION BETWEEN *IN VIVO* AND *IN VITRO* STUDIES

In seven patients the results of *in vivo* and *in vitro* studies could be compared and their concordance evaluated (Table 3). In patients treated with 4-aminoquinolines (chloroquine or amodiaquine) a good correlation was seen, as resistance was noticed in all patients both *in vivo* or *in vitro*. In four patients treated with quinine that could have their results compared, good correlation was seen in three, in that parasites were sensible to the drug both *in vivo* and *in vitro*. In one patient however an *in vivo* R III response was seen, in spite of *in vitro* sensitivity. The overall kappa statistic for agreement between *in vivo* and *in vitro* tests was 0.72 in the present study, thus demonstrating substantial correlation between these studies of *Plasmodium falciparum* chemoresistance to antimalarials.

TABLE 3

Correlation of *in vivo* and *in vitro* studies for *Plasmodium falciparum* resistance to chloroquine, amodiaquine and quinine

***In vivo* studies**	***In vitro* sensivity microtests**		**Total**
	Sensitive strains	**Resistant strains**	
Sensitive strains	3	0	3
Resistant strains	1	3	4
Total	4	3	7

Kappa statistic = 0.72

DISCUSSION

Effective control of malaria transmission in a given area requires the reasonable use of antimalarial drugs in an attempt to eliminate the only reservoir of *Plasmodium falciparum*, that is infected individuals. Therefore defining the precise distribution of drug resistant strains and assessing the extent of chemoresistance is important in the establishment of efficacious chemotherapy.

Distinguishing chemoresistance from treatment failure due to other reasons requires the performance of *in vivo* or *in vitro* studies of parasite sensitivity to antimalarials. However such studies may be hindered in endemic areas by the fact that reinfections cannot be ruled out in areas with continuous malaria transmission. The present study was then designed to be conducted in a non-endemic area in order to avoid such limitations. On the other hand reduction in the total number of followed individuals was expected by the authors, as patients whith malaria when diagnosed in Brazilian non-endemic areas usually which to be treated promptly in order to return home as soon as possible.

No statistically significant difference was noticed between patients in Groups A and B, concerning initial parasitemia and drug absorption. Therefore any differences in treatment outcome

could be attributed to chemoresistance. Parasite sensitivity to antimalarials was remarkably distinct in patients treated with the different antimalarial drugs. High degree of resistance to 4-aminoquinolines (chloroquine and amodiaquine) was seen in this study in concordance to results of other investigators[3, 10]. However even though chemoresistance to 4-aminoquinolines was the rule, patients in our study exhibited R I and R II responses, what indicates partial relief of symptoms after drug intake. Similar patterns of response to chloroquine treatment were described in Maranhão[28] and Amazonas[4]. These results support the concept that chemotherapy with 4-aminoquinolines may still have a role to play in the treatment of semi-immune patients with falciparum malaria in the Amazon region, reducing its morbidity and mortality when other more active drugs are unavailable or their use unfeasible, as for instance in remote areas. On the other hand the high *in vitro* resistance to chloroquine shown in the present study is concordant to previous reports from the Brazilian Amazon region [2, 3, 12, 26, 31].

No advantage was detected in the use of amodiaquine instead of chloroquine, as has been advocated by other studies in Thailand[15, 30], Kenya[29], West Africa[24], and Central Africa[8]. In the Brazilian Amazon region cross-resistance among 4-aminoquinolines seems to be the rule[17, 19] and may be attributed to the widespread use of amodiaquine in our country, as an alternative to chloroquine, after resistance to the latter was repeatedly identified.

Quinine in our cohort was effective in the treatment of 80% of patients. In one instance however, progressive increase in asexual parasitemia after treatment, in spite of positive urinary excretion test for the drug, evidenced an *in vivo* R III response. However incomplete drug absorption could not be ruled out in this occasion, as the assessment of serum quinine concentrations was not available for the present investigation. Anyhow clinical deterioration of the patient required his exclusion from the study and the introduction of alternative chemotherapy, that was indeed successful. Complete clearance of his asexual parasitemia was obtained with intravenous quinine plus oral mefloquine.

Resistance of *Plasmodium falciparum* to quinine was originally described in Brazil in the beginning of this century[11, 21]. Moreover new evidence of *in vivo* resistance to this compound has been more recently demonstrated[1,7], while the same trend is being reported from Asia[5]. *In vitro* resistance to quinine had been previously reported in Brazil[6, 26], but our data may support the idea that its importance is increasing in our country, as previously proposed by COUTO et al.[10] when the authors monitored *in vitro* resistance to this antimalarial agent in an Amazon location in two different time points. In East Africa R III *in vivo* responses after chemotherapy of falciparum malaria with quinine have been correlated to *in vitro* evidence of resistance of *P. falciparum* to this drug[16].

Probit analysis of log dose-response to antimalarials in the present study yielded extremely high inhibitory concentrations of 4-aminoquinolines (choroquine and amodiaquine), suggesting high *in vitro* resistance to these compounds. Likewise high inhibitory concentratons were calculated for quinine as well.

An overall substantial correlation between *in vivo* and *in vitro* tests for assessment of *Plasmodium falciparum* sensitivity or resistance to antimalarials was evidenced in the present study (kappa statistic = 0.72). Full concordance between *in vivo* and *in vitro* analysis of chemoresistance to 4-aminoquinolines was shown. However in the group of patients treated with quinine one discordant result was seen. Even though *in vivo* chemotherapy was unsuccessful in this patient, *in vitro* resistance of *P. falciparum* could not be demonstrated in the quinine sensitivity microtest. As serum antimalarial concentrations were not assessed in our study, one might still consider incomplete drug absorption or insufficient dosage to have occurred. Moreover *in vivo* selection of resistant strains after treatment could also have happened, what would not have been detected in the *in vitro* microtest, as this technique involves a single parasite schizogonic cycle.

The present results thus emphasize chemoresistance of *P. falciparum* to chloroquine, amodiaquine and quinine in Brazil as an important cause of therapeutic failure to conventional antimalarial drug regimens and reinforce the need for continuous monitoring of drug resistance in different areas of the country as an essential tool for the correct definition of malaria chemotherapy.

RESUMO

Resistência *in vivo* e *in vitro* do *Plasmodium falciparum* à cloroquina, amodiaquina e quinino na Amazônia Brasileira.

Com o propósito de avaliar a resistência do *Plasmodium falciparum* às drogas antimaláricas, rotineiramente empregadas no Brasil, os autores acompanharam dez pacientes com malária falciparum adquirida na Amazônia brasileira. Os pacientes foram submetidos a estudo *in vivo* de sensibilidade a drogas, após tratamento com derivados 4-aminoquinoleínicos (cloroquina e amodiaquina) ou quinino. A absorção das drogas foi verificada através de testes padronizados de excreção urinária de antimaláricos. Oito pacientes puderam ser seguidos por 28 dias. Dentre eles detectou-se resistência *in vivo* (em nível de R I e R II) em todos os pacientes tratados com 4-aminoquinoleínas. Um paciente tratado com quinino exibiu padrão de resistência R III ao tratamento.

Alíquotas de sangue periférico dos mesmos pacientes foram ainda submetidas a microtestes *in vitro* de sensibilidade a antimaláricos. Em nove microtestes houve desenvolvimento satisfatório de esquizontes. Destes detectou-se resistência *in vitro* à cloroquina e amodiaquina em 100% e ao quinino em 11,11% das amostras. Através da análise de probitos calcularam-se as concentrações de cloroquina, amodiaquina e quinino, capazes de inibir o crescimento *in vitro* de esquizontes (Cl_{50}, Cl_{90} e Cl_{99}). Em seis pacientes observou-se concordância entre os achados *in vivo* e *in vitro*. Os resultados ressaltam o alto grau de resistência do *P. falciparum* às 4-aminoquinoleínas e indicam um aumento na resistência do parasita ao quinino na Amazônia brasileira. Reitera-se, assim, a necessidade de monitoração contínua da sensibilidade a antimaláricos, com o intuito de recomendar esquemas mais eficazes na terapêutica da malária por *P. falciparum*.

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