Assessment of Three In Vitro Tests and an In Vivo Test for Chloroquine Resistance in Plasmodium falciparum Clinical Isolates

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Three in vitro assays (the isotopic semimicrotest [700 µl per well; 24-well plates], the isotopic microtest [200 µl per well; 96-well plates], and the rapid in vitro test) and the standard in vivo test for chloroquine resistance were compared for 99 clinical isolates of Plasmodium falciparum obtained from symptomatic African patients. The 50% inhibitory concentrations determined by the two isotopic tests were similar and were highly correlated (r = 0.965; P < 0.05), showing a high concordance between the semimicrotest and the microtest. There was a moderate agreement between these two isotopic tests and the in vivo test. Most of the discordant results were probably due to host factors, including reinfections, pharmacokinetic variations, and immunologic response, which are eliminated in in vitro assays. The rapid in vitro test based on the inhibition of chloroquine efflux in the presence of verapamil was poorly concordant with the other tests. Despite some discordant results, isotopic in vitro assays are useful to characterize the phenotypes of individual isolates without the interference of host factors and are complementary to in vivo evaluation of drug efficacy. However, in vitro assays need to be standardized to allow direct comparison of results between different laboratories.

Malaria infection due to Plasmodium falciparum is one of the major potentially fatal parasitic diseases that are endemic in many tropical and subtropical regions of the world. Chloroquine has been the drug of choice for both chemoprophylaxis and treatment of P. falciparum malaria for several decades, but its clinical utility has been greatly reduced in many regions where malaria is endemic due to the spread of chloroquine resistance (26–29). Because chloroquine is well tolerated, safe for pregnant women and young children, cheap, efficacious, and widely available, its clinical use needs to be carefully monitored. This is especially true in most of sub-Saharan Africa, where chloroquine still remains the mainstay of malaria treatment for indigenous populations (30). The rapidly changing epidemiology of chloroquine resistance requires an accurate tool to follow the evolution of drug resistance.

The existing tools include in vitro and in vivo tests. There are three major types of in vitro tests used today: the isotopic microtest, the isotopic semimicrotest, and the World Health Organization (WHO) microtest, which requires microscopic examination for determination of the parasite count (6, 8, 19). All of these assays involve the determination of the drug concentration at which parasite growth is inhibited over a 24- to 48-h incubation period. Unlike the in vivo test, in vitro assays have not been standardized and yield results which are not directly comparable. The in vivo test follows the clinical and parasitological responses in symptomatic patients over a period of time (usually 14 or 28 days) after drug therapy. The in vivo tests have been standardized by the WHO (6, 25). Another in vitro assay, referred to as a “rapid in vitro test” in the present paper, has been described by Gluzman et al. (11). The principle of a rapid in vitro test is based on two observations: first, chloroquine-resistant parasites actively expel chloroquine while chloroquine-sensitive parasites accumulate the drug (12), and second, the addition of verapamil inhibits chloroquine efflux in chloroquine-resistant parasites but not in chloroquine-sensitive parasites (15). Thus, if a given P. falciparum isolate incorporates similar quantities of radiolabeled chloroquine with or without verapamil, the isolate is chloroquine sensitive. On the contrary, if an isolate incorporates more chloroquine in the presence of verapamil, the isolate is chloroquine resistant.

So far, there have been no reliable data comparing the results of these various tests for chloroquine resistance in field isolates. Our aim was to determine to what extent the isotopic semimicrotest, isotopic microtest, rapid in vitro test, and in vivo test were concordant by using P. falciparum clinical isolates obtained from Cameroonian patients whose chloroquine response was evaluated in parallel with these in vitro assays.

MATERIALS AND METHODS

Patients. The study was part of a clinical trial comparing chloroquine and pyronaridine in Yaoundé, Cameroon (20). Ninety-nine symptomatic, malaria-infected Cameroonian adults (n = 62; male/female ratio, 29/33; age range, 15 to 45 years) and children (n = 37; male/female ratio, 16/21; age range, 5 to 14 years) were enrolled in the study. Participants had to fulfill the following criteria: show signs and symptoms of acute uncomplicated falciparum malaria (a fever of >37.5°C on enrollment or history of fever within the past 24 h), mono-infection with P. falciparum, initial parasitemia of >5,000 asexual parasites per µl of blood, and a negative Saker-Solomons urine test for anti-malarial drugs (16). Pregnant women, patients with signs and symptoms of severe and complicated malaria as defined by the WHO (23), and patients with severe anemia (hemoglobin <6 g/dl) were excluded. The study was approved by the Cameroonian National Ethics Committee.

Drugs. Chloroquine sulfate was provided by Rhône-Poulenc-Rorer (Anthony, France). A stock solution of chloroquine was prepared in sterile distilled water. Twofold serial dilutions of the drug were made in sterile distilled water (19, 20), and the final concentrations ranged from 25 to 1,600 nmol/liter. Each concentration was distributed in triplicate in 24- or 96-well tissue culture plates and air dried. [3H]Chloroquine (specific activity, 25.5 Ci/mmol; New England Nuclear, Boston, Mass.) was a gift from D. J. Krogstad. A solution with 250 nM [3H]chloroquine was prepared in RPMI 1640 medium for use in the rapid in vitro assay.

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Verapamil hydrochloride was obtained from Sigma Chemical Co. (St. Louis, Mo.).

In vivo test. The patients received a total of 25 mg of base of chloroquine sulfate tablets/kg of body weight in three divided doses (10 mg of base on days 0 and 1, and 5 mg base on day 2) under supervision. As recommended by the recent WHO protocol (25), the patients were followed on an outpatient basis on days 1, 2, 3, 4, 7, and 14. At each visit, the patients’ clinical conditions, body temperatures, and parasitemia were assessed. Parasite density was determined by counting the number of infected erythrocytes per 20,000 erythrocytes in Giemsa-stained thin blood films (on day 0) or the number of asexual parasites per 1,000 leukocytes in Giemsa-stained thick blood films (from day 1 onwards) and was expressed as the number of asexual parasites per microliter of blood. This conversion was calculated from the complete blood count of each patient on day 0. Parasite density in thin films was initially expressed as the percentage of infected erythrocytes among 20,000 erythrocytes, and this percentage and the erythrocyte count per microliter of the patient’s blood on day 0 were multiplied. Likewise, the number of asexual parasites per 1,000 leukocytes in thick films was multiplied by the number of leukocytes per microliter of blood on day 0 to determine the parasite density.

Isotopic semimicrotest and microtest. Venous blood samples (5 to 10 ml) were collected in a tube coated with EDTA before treatment. Infected erythrocytes were washed three times in RPMI 1640 culture medium. The erythrocytes were resuspended in the complete RPMI 1640 medium, consisting of 10% human serum (obtained from European blood donors without previous history of malaria), 25 mM of HEPES buffer, and 25 mM of NaHCO₃/L at a hematocrit of 1.5% and an initial parasitemia of 0.2 to 1.0%. If the blood sample had a parasitemia of >1.0%, fresh, unfixed erythrocytes were added to adjust the parasitemia to 0.6%. For the semimicrotest, 700 µl of the suspension of infected erythrocytes was distributed in each well of the 24-well tissue culture plates (19). For the microtest, 200 µl of the suspension was distributed in 96-well tissue culture plates (8). The parasites were incubated at 37°C in 5% CO₂ for 18 h. To assess parasite growth, [3H]hypoxanthine (specific activity, 5 Ci/mmol, 1 µCi/well) (Amersham, Buckinghamshire, United Kingdom) was added after the first 18 h of incubation. After an additional 24 h of incubation, the plates were frozen to terminate the in vitro drug assays. The plates were thawed to lyse infected erythrocytes, and the contents of each well were collected on glass fiber filter papers, washed, and dried with a cell harvester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillant; Amersham) was added (19). A liquid scintillation counter (Wallac 1410; Pharmacia, Upplusa, Sweden) was used to quantitate the incorporation of [3H]hypoxanthine.

Rapid in vitro test. The essential procedures of the rapid in vitro test were described previously (11). The 1994 modified protocol was provided through the courtesy of D. J. Krogstad. Briefly, infected erythrocytes were suspended in RPMI 1640 culture medium (1:14 [vol/vol] for parasite densities of >20,000/µl; 1:4 for parasite densities of 5,000 to 20,000/µl), and 150 µl of the suspension was mixed with an equal volume of RPMI 1640 medium with or without 25 µM of verapamil/liter (final concentration, 10 µM/liter) and 75 µl of medium containing 200 to 500 µM of [3H]chloroquine/liter (final concentration, 50 nM/liter). After incubation for 1 h at 37°C, three 100-µl samples were transferred from each tube, with or without verapamil, into microcentrifuge tubes containing silicon oil and centrifuged to separate the erythrocytes from the medium. The erythrocyte pellets were cut from the tube, digested with Protosol-ethanol mixture (75 µl; 1:2 [vol/vol]) (New England Nuclear) for 1 h at 35°C, decolorized with 25 µl of 30% H₂O₂, and acidified by adding 25 µl of 1 N HCl. The treated pellets were transferred into scintillation vials with 10 ml of scintillation cocktail (Unicocktail; ICN Radiochemicals, Irvine, Calif.) and measured for tritium in a liquid scintillation counter.

Interpretation of results. For the isotopic semimicrotest and microtest, the logit of parasite growth inhibition was determined from the level of [3H]hypoxanthine accumulation and plotted against the logarithm of concentrations. A linear regression analysis was used to calculate the 50% inhibitory concentration (IC₅₀), defined as the drug concentration resulting in 50% of the uptake of [3H]hypoxanthine seen in the drug-free control wells. The threshold IC₅₀ was set at 100 nmol/liter (19).

For the rapid in vitro test, the difference in chloroquine accumulation with and without verapamil was expressed as the percentage of chloroquine accumulation calculated with the following formula: counts per minute with verapamil minus counts per minute without verapamil divided by minutes per count minus without verapamil. Chloroquine sensitivity and chloroquine resistance were defined as the percentage of change <−10% and >+20%, respectively. Values between −10 and +20% were interpreted as low-level resistance and were grouped together with chloroquine-resistant isolates (11).

The therapeutic response of patients treated with chloroquine was graded as follows: parasitological response A, a negative thick blood smear or a positive smear with a density <25% of the initial density on day 3 and negative smears until day 14; response B, a positive smear on days 3 and 7 (a density <25% of the initial density on day 0) and 7 or the requirement for an alternative antimalarial drug between days 3 and 7 due to worsening of clinical conditions; parasitological response C, a positive smear on day 3 (≥25% of the density on day 0) for alternative antimalarial therapy on or before day 3 (25). For data analysis, the in vivo response was classified as S (sensitive) if the parasitological response was A and R (resistant) if the parasitological response was B or C.

Statistical analysis. Interpretable results of the isotopic semimicrotest and microtest were defined as an adequate incorporation (>threefold difference) of the radiolabeled hypoxanthine in the drug-free control wells compared with the incorporation in the wells containing 1,600 nmol of chloroquine/liter. In vivo tests were defined as an adequate incorporation fulfilling the incubation and the 14-day follow-up with regular clinical and parasitological evaluations on days 1, 2, 3, 7, and 14. Uninterpretable results were excluded from data analysis.

The IC₅₀ determined from the semimicrotest and microtest were compared by the paired t test. The level of significance was set at 0.05. A χ² test was used to determine whether the proportions of chloroquine-resistant isolates were similar with the different assays. The correlation coefficient between the IC₅₀ determined by the semimicrotest and microtest was calculated by linear regression analysis. For qualitative values, the kappa coefficient of Cohen was calculated to assess the degree of agreement between various tests of resistance (10).

The levels of agreement were arbitrarily classified as follows: 0 to 0.20, slight agreement; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, good agreement; and >0.81, very good agreement. For quantitative comparison of IC₅₀ determined by the semimicrotest and microtest, the coefficient of interclass correlation (r) was calculated by one-way analysis of variance (9).

RESULTS

The semimicrotest and rapid in vitro test were performed against 99 field isolates; the microtest was performed with 60 isolates of the chloroquine-resistant patients from whom the isolates were obtained, 61 were randomly assigned to chloroquine treatment. The other 38 patients were treated with other antimalarial drugs. The proportions of interpretable in vitro tests were 92 of 99 (93%) for the semimicrotest, 57 of 60 (95%) for the microtest, and 99 of 99 (100%) for the rapid test. For the in vivo test, 52 of 61 (85%) chloroquine-treated patients were followed until day 14; 9 patients were lost to follow-up.

The distribution of the IC₅₀ determined by the semimicrotest and microtest was presented in Fig. 1. The results of the semimicrotest showed that the geometric mean IC₅₀ of the chloroquine-sensitive isolates and the chloroquine-resistant isolates (95% interval of confidence) were 30.1 nmol/liter (25.8 to 35.3 nmol/liter) and 231 nmol/liter (198 to 269 nmol/liter), respectively. According to the microtest, the geometric mean IC₅₀ were 30.1 nmol/liter (23.2 to 39.0 nmol/liter) for the chloroquine-sensitive isolates and 220 nmol/liter (179 to 270 nmol/liter) for the chloroquine-resistant isolates. There was no statistical difference (P > 0.05) between the IC₅₀ determined by the two isotopic in vitro tests, and the IC₅₀ were highly correlated (r = 0.965; P = 0.0001; n = 57).

FIG. 1. Distribution of the IC₅₀ determined by the semimicrotest and microtest. The IC₅₀ determined by these tests were similar and were highly correlated (r = 0.965; P < 0.05; n = 57).
Of the interpretable results, 42 of 92 (46%), 28 of 57 (49%), 35 of 99 (35%), and 24 of 52 (46%) were resistant to chloroquine according to the semimicrotest, microtest, rapid in vitro test, and in vivo test, respectively (Fig. 2). The similar proportions, as determined by the $\chi^2$ test ($P > 0.05$), of the chloroquine-resistant \textit{P. falciparum} isolates were not due to concordant results. A “very good” agreement was observed between the semimicrotest and microtest ($\rho = 0.96$ [Table 1]). A “moderate agreement” was observed between the in vivo test and semimicrotest (kappa = 0.48) or microtest (kappa = 0.52). There was “slight agreement” between the rapid in vitro test and semimicrotest (kappa = 0.07), microtest (kappa = 0.09), and in vivo test (kappa = 0.05).

**DISCUSSION**

Our comparative study shows that the microtest and semimicrotest give equivalent results qualitatively and quantitatively. A preference for either one of the isotopic in vitro tests should therefore be based on factors other than their accuracy. The microtest has major advantages over the semimicrotest, including the possibility of performing at least part of the test automatically and the use of a smaller quantity of material. The latter advantage represents an important gain in laboratories that perform the test on a routine basis, since standard in vitro tests require expensive material, such as 10% type AB human serum in the culture medium. A smaller amount of patients’ venous blood (less than one-third of the volume) is also required for the microtest, which facilitates an in vitro study on isolates obtained from small children. In addition, four 24-well plates are necessary to carry out the same number of tests as in a single 96-well plate. These advantages, as well as the similar results obtained with the two isotopic tests, have led us to abandon the semimicrotest, and our laboratory work is now entirely based on the isotopic microtest.

Although the rapid in vitro test was developed on a rational basis involving the inhibition of an active chloroquine efflux by verapamil in resistant parasites, in practice, this test failed to distinguish between chloroquine-sensitive and chloroquine-resistant isolates, as determined by either isotopic in vitro tests or the in vivo test. Several technical faults may be the causes. Firstly, most fresh clinical isolates are synchronous and are at the young ring stage at the time of blood extraction. Incubation of these ring forms for 1 h is probably insufficient to detect parasite metabolism, as the ring stage lasts 12 to 24 h in \textit{P. falciparum} (13). Various experimental works have shown that ring stages are metabolically the least active (5, 18, 21). Other studies have shown that most antimalarial drugs, including chloroquine, exhibit stage-dependent activity and that peak activity is usually observed during the trophozoite and early schizont stages (31). These experimental results imply that fresh clinical isolates should be incubated with or without verapamil for at least 12 to 24 h before a clear distinction between the chloroquine-sensitive and chloroquine-resistant parasites can be made. Secondly, the viability of fresh isolates is not assessed by the rapid in vitro test, as is shown by the discrepancy in interpretable results between the rapid and isotopic tests. A longer incubation time with the isotopic in vitro tests revealed that not all isolates are viable, as reflected by a poor incorporation of radiolabeled DNA precursor. The possible
reasons why some isolates are not viable in vitro include self-medication before consultation that is undetected by a standard urine test; unexplained factors that transform in vitro most of the ring forms into gametocytes, which do not undergo nuclear division and thus do not incorporate a significant amount of radiolabeled DNA precursor; and serum factors that inhibit parasite growth. Thirdly, the rapid in vitro test is probably not sensitive enough to detect the difference in chloroquine incorporation in chloroquine-resistant isolates when the parasitemia is below 1%. Chloroquine is known to accumulate in uninfected erythrocytes, leading to diminished sensitivity of the rapid in vitro test at low parasitemias (22). Initial experiments were done with culture-adapted parasites at 1% parasitemia (11). The data obtained by Gluzman et al. do not display distinct differences between the chloroquine-sensitive and the chloroquine-resistant \textit{P. falciparum} strains below 1% parasitemia. Since a large majority of malaria-infected patients present with a parasitemia of <1%, the rapid test may not be suitable for clinical isolates. In addition, the threshold values for chloroquine resistance based on laboratory-adapted \textit{P. falciparum} clones may not be applicable to the parasite isolates. The above-mentioned technical considerations, as well as the poor performance of the rapid in vitro test compared with the isotopic in vitro tests and the in vivo test, preclude any practical application of the rapid in vitro test in clinical isolates without further technical improvements. However, the rapid in vitro test may be considerably improved and may prove to be an accurate diagnostic tool for chloroquine resistance if the incubation period is extended to 12 to 24 h, or even to 48 h. Previous in vitro studies have shown that chloroquine-sensitive and chloroquine-resistant fresh isolates of \textit{P. falciparum} can be distinguished by the property of resistance modulators (e.g., verapamil, amiodipine, desipramine, cyproheptadine, chlorpheniramine, and chlorpromazine) to decrease the level of resistance in chloroquine-resistant parasites but not in chloroquine-sensitive isolates (1–4). These studies were designed to determine the IC\textsubscript{50}, requiring the standard incubation time of 42 to 48 h to allow parasite maturation to the schizont stage in test plates with a full range of chloroquine concentrations. The obvious drawback to this proposed technical modification is the loss of rapidity in obtaining the results.

Concordance between the isotopic in vitro tests and the in vivo test was moderate. Several factors explain why the level of concordance was not higher, as was expected. Parasite clearance in malaria-infected patients depends on various pharmacodynamic and pharmacokinetic parameters, and the level of acquired immunity interacts with and enhances drug efficacy (17). A patient harboring chloroquine-resistant populations of \textit{P. falciparum}, as determined by an in vitro test, may thus eliminate all parasites after an adequate treatment with chloroquine, due to the “booster effect” of the immune system. A patient infected with chloroquine-sensitive parasites, on the other hand, may fail to clear the parasites within 14 days because of an inadequate plasma chloroquine level or reinfec tion a few days before or after chloroquine treatment is administered. In the latter case, the new populations of chloroquine-sensitive or chloroquine-resistant parasites may emerge in the peripheral blood circulation when a subtherapeutic plasma chloroquine level has been attained before day 14. It is important to note that the in vivo test measures the proportion of therapeutic failure in a given patient population, which may or may not be directly related to drug resistance, while in vitro tests measure the capacity of parasites to grow under different concentrations of drugs. Isotopic in vitro tests seem to be more objective and more accurate in characterizing the phenotype of parasites, independently of various host factors that may render the interpretation of in vivo tests difficult.

In this study, we have compared three different in vitro assays and an in vivo test and have observed a near-perfect concordance between two isotopic (semimicrotest and microtest) in vitro assays, a moderate agreement between these two isotopic in vitro tests and the in vivo test, and no significant agreement between the rapid in vitro test and any of the other tests of resistance. The isotopic in vitro tests seem to be the most suitable methods to characterize the phenotype of parasites without any interfering host factors. Alternative in vitro assays, such as those based on parasite lactate dehydrogenase activity, may also be useful (5, 14). However, because of the proliferation of variant in vitro assays, the parameters used in these assays, including hematocrit, volume per well, parasitemia, and serum (or its substitutes) composition, need to be standardized to yield directly comparable results, as is the case with antibiotic sensitivity tests for bacteria (6–8, 19, 24). Since the degree to which pharmacologic and immunologic host factors interfere with in vivo tests varies in different study populations, the isotopic in vitro tests are best performed concomitantly with the in vivo test in the field for the complete epidemiological description of field isolates in a given area of endemicity.

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