Plasmodium falciparum Malaria: Rosettes are Disrupted by Quinine, Artemisinin, Mefloquine, Primaquine, Pyrimethamine, Chloroquine and Proguanil

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An assay was developed measuring the disruption of rosettes between Plasmodium falciparum*-infected (trophozoites) and uninfected erythrocytes by the antimalarial drugs quinine, artemisinin mefloquine, primaquine, pyrimethamine, chloroquine and proguanil. At 4 hr incubation rosettes were disrupted by all the drugs in a dose dependent manner. Artemisinin and quinine were the most effective anti-malarials at disrupting rosettes at their therapeutic concentrations with South African RSA 14, 15, 17 and The Gambian FCR-3* P. falciparum *strains. The least effective drugs were proguanil and chloroquine. A combination of artemisinin and mefloquine was more effective than each drug alone. The combinations of pyrimethamine or primaquine, with quinine disrupted more rosettes than quinine alone. Quinine may be an effective drug in the treatment of severe malaria because the drug efficiently reduces the number of rosettes.*

Key words: malaria - *Plasmodium falciparum* - rosette disruption - antimalarial drugs

Plasmodium falciparum infected erythrocytes at the trophozoite stage of parasite development adhere to uninfected erythrocytes forming "rosettes" (David et al. 1988). This adherence probably enhances the ability of schizonts when rupturing the host cell to invade the surrounding naive red cells. In studies with field strains from Africa and Madagascar, there appears to be a link between the number of rosettes formed from a strain, the severity of the disease in the patient and the presence of anti-rosetting antibodies, implying a protective role for the anti-rosetting antibodies (Carlson et al. 1990a, Treutiger et al. 1992, Ringwald et al. 1993, Rowe et al. 1995). In contrast, rosette disrupting antibodies do not seem to protect adults or children against cerebral malaria in Papua New Guinea (Rogerson et al. 1996).

Rosetting only occurs as the parasites develop into trophozoites suggesting that the ligands are either alterations to host surface antigens or novel parasite antigens. Ligands that have been implicated include a histidine rich protein (Carlson et al. 1990b), low molecular mass proteins called "rosettins" (Helmby et al. 1993), parasite variant erythrocyte membrane protein 1 (Rowe et al. 1997, Chen et al. 1998), complement receptor 1 (Rowe et al. 1997) and lectins (Carlson & Wahlgren 1992). Rosettes can be disrupted by anti-histidine rich protein 1 antibodies (Carlson et al. 1990b), antibodies from malaria patients (Treutiger et al. 1992), and sulphated glycoconjugates (Rogerson et al. 1994, and Rowe et al. 1994).

We have added seven antimalarial drugs to four strains of *P. falciparum* which form rosettes in culture and found that each drug disrupted rosettes in a dose dependent manner. Combinations of two drugs together disrupted more rosettes than each drug on its own.

MATERIALS AND METHODS

P. falciparum cultures - The Gambian FCR-3 and the South African RSA 14, 15 and 17 (Freese et al. 1991) *P. falciparum* strains were maintained in continuous *in vitro* culture according to the methods of Trager and Jensen (1976) as modified by Freese et al. (1988). Briefly, parasitised O^+ human erythrocytes (FCR-3) and A^+ (RSA), were cultured at 37°C in medium consisting of 10.41 g/l RPMI 1640 powdered medium supplemented with 5.94 g/l HEPES buffer, 50 mg/l gentamicin, 44 mg/l hypoxanthine, 4 g/l sucrose, 5 g/l sodium bicarbonate and 10% heat-inactivated human AB serum made to 1 litre with sterile water and gassed with CO_2 until the medium changed colour from

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Received 30 November 1998
Accepted 7 June 1999

red to yellow. Parasites were synchronised at ring stages using sorbitol (Lambross & Vanderberg 1979) and growth monitored by microscope examination of Geimsa stained parasites. Chemicals, quinine, artemisinin chloroquine, primaquine (diphosphate salt), pyrimethamine and media were all purchased from Sigma chemical company, St Louis, MO, USA. Mefloquine was obtained from Roche Products (Pty) Ltd and proguanil hydrochloride from Zeneca, Johannesburg, South Africa. Blood from healthy donors was obtained from the Johannesburg Blood Transfusion Service, Johannesburg, South Africa.

Rosetting assay - When a parasitaemia of 8-10% was reached and the parasites were at the trophozoite-schizont stage, rosette formation was evaluated based on the method described by Carlson et al. (1990b). An aliquot of the parasite culture was mixed with one tenth volume of an acridine orange solution (0.001 %) and incubated for about 5 min at room temperature. The cells were mounted onto a slide with a coverslip. Rosettes were counted over consecutive fields with a 40x fluotar lens in incident ultraviolet light using a Dialux 20 fluorescent microscope.

Rosette formation was characterised by a parasitised red blood cell that had bound two or more uninfected red blood cells. On average, 250 consecutive parasitised erythrocytes were counted and rosettes evaluated. The effect on rosette formation by drugs was expressed as a percent disruption of the control and calculated using the following formula: % disruption = 1 - [rosetting after treatment/rosetting in untreated control] x 100.

Effect of drugs on parasite rosetting - Aliquots of parasite culture were incubated with each of the antimalarials prepared at therapeutic concentrations as indicated in the appropriate references. These concentrations were: 0.2 µg/ml for chloroquine, 0.153 µg/ml for primaquine, 155 ng/ml for pyrimethamine (Desjardins et al. 1988), 20 µg/ml for quinine (Peters 1987), 1 µg/ml for mefloquine (Slutsker et al. 1990), 1µg/ml for proguanil (Taylor et al. 1990) and 400 ng/ml for artemisinin (Duc et al. 1994). In the first experiment parasites were incubated from rings to trophozoites with each of the drugs and then rosettes enumerated. In the second experiment, the drugs were added to a trophozoite stage culture over a 6 hr period and rosettes counted at different time points.

The effects of different concentrations of antimalarial drugs on rosettes - Parasites at late trophozoite stage (8-10% parasitaemia, 1-2% haematocrit) were incubated with each of the seven drugs for 4 hr before rosettes were counted. The concentrations used spanned the therapeutic range of each drug and were over the following ranges as indicated on figures: quinine from 5 µg to 33 µg; artemisinin from 100 ng to 800 ng; mefloquine from 0.2 µg to 2.1 µg; primaquine from 4 ng to 28 ng; pyrimethamine from 50 ng to 320 ng; chloroquine from 50 ng to 425 ng; proguanil from 0.3 µg to 2.0 µg.

Effect of combinations of two drugs on rosetting - Artemisinin or mefloquine were added to parasites at late trophozoites (8-10% parasitaemia). In turn one drug was at the therapeutic concentration and the concentration of the other drug varied. In a second experiment therapeutic concentrations of combinations of quinine, chloroquine, pyrimethamine and primaquine were incubated with rosetting parasites at 37°C for 4 hr and rosettes counted.

Two controls were used in every experiment. A sample of the malaria culture was removed at the start of the experiment and cultured in the absence of drugs under identical conditions to experiments with drugs. At the end of the appropriate times half of the control was treated with the pertinent drug for 5 min and rosetting enumerated. The second half of the sample was evaluated without any additional treatment. Both controls produced the same number of rosettes. All the above experiments were conducted under identical conditions of pH, ionic concentration, carbon dioxide concentration and temperature.

RESULTS

Initially we determined that incubating each antimalarial drug with a malarial culture during parasite development from rings to trophozoites lead to reduced rosette formation. We found that the optimum time to determine the perturbation of rosettes was when the drugs were incubated for 4 hr with parasites at the trophozoite stage. At this stage rosettes are already present in the culture. A 4 hr time frame has been used to evaluate the effects of glycoconjugates and enzymes on rosetting (Rowe et al. 1994) and a 4 hr time frame has been shown to be sufficiently long for ligands which have been removed by enzymatic cleavage to be resynthesised (Hommel & Semoff 1988). We therefore adopted a 4 hr assay for all our subsequent experiments. In all experiments we ran two controls, one was a drug free culture sample of the cultures incubated under identical conditions and the second was to take the drug free culture and add drugs at the end of the assay. Both controls produced identical numbers of rosettes.

To confirm that the effect of drugs on rosetting depended on the concentration of each drug being used, a range of drug concentrations above and below the therapeutic doses was added to rosetting parasites and the rosettes counted 4 hr later. All

the drugs (Fig.1A-G) disrupted rosettes formed by the four strains in a dose dependent manner. The most effective drugs appear to be quinine (Fig.1A) and artemisinin (Fig.1B) at both the therapeutic concentration and the highest concentration tested. Mefloquine (Fig.1C), primaquine (Fig.1D) and pyrimethamine (Fig. 1E) have similar characteristics, though mefloquine is comparably more effective at therapeutic concentrations than the highest concentration used. The least effective drugs are chloroquine (Fig. 1D) and proguanil (Fig.1E). The order of drugs disrupting rosettes i.e. most effective to least effective, was the same, whether considering the South African strains alone or including data for the FCR-3 strain. At concentrations of each drug predicted to occur in the plasma of patients (therapeutic concentrations), all the drugs disrupted rosettes. The four *P. falciparum* strains each exhibit a unique and specific profile of sensitivity to disruption by each drug (Fig.1A-G).

In the presence of a combination of the therapeutic dose of artemisinin with a range of concentration of mefloquine or, alternately, the therapeutic dose of mefloquine and varying the concentration of artemisinin there was increased rosette disruption with an increase in drug concentrations (Fig. 2). Keeping mefloquine concentrations constant and increasing artemisinin concentrations was more effective than maintaining artemisinin and increasing mefloquine concentrations. This is consistent with the results above obtained for each drug alone.

When quinine, pyrimethamine, chloroquine and primaquine were combined, some combinations were more effectual at disrupting rosettes of the FCR-3 strain than others (Table). Rosette disturbance ranged from 35% to 82%. Drug combinations with chloroquine exhibit the lowest percent disturbance of rosetting while combinations with quinine were the most effective.

DISCUSSION

It was consistently observed that chloroquine and primaquine were the least productive at disrupting and preventing rosette formation with the four *P. falciparum* strains at both the therapeutic and maximum doses tested. Concentrations of each drug at and around reported concentrations found in patient plasma (therapeutic concentrations) were chosen to ensure that experiments approximated physiological conditions predicted to occur in host blood during treatment with anti-malarial drugs. This necessitated using different concentrations for each drug, and the axis for each figure is therefore different. Direct comparisons between concentrations of drugs are only possible at the therapeutic concentrations of the drugs. The most effective drugs were quinine and artemisinin (Figs 1A, B). The laboratory strains, RSA 14, 15 and 17 and FCR-3 used in the present study have been characterized as chloroquine resistant (Freese et al. 1991) and we obtained similar results repeating that study. It would appear that these strains which are resistant to chloroquine, have fewer of their rosettes disrupted by the drug.

Of all the drugs tested, quinine and artemisinin were the most impressive at reducing the number of rosettes. This was true at concentrations from the therapeutic dose to the highest dose of the drugs tested. Artemisinin has been reported to have a fast parasite clearance time (Jaing et al. 1982), and quinine is the current drug of choice for the treatment of cerebral malaria. Artemether, a derivative of artemisinin has been studied as a replacement for quinine in the treatment of cerebral malaria. One study found that artemether is more effective than quinine and improves the coma resolution time in children in a Malawian study (Taylor et al. 1993). In contrast in a similar study in Kenya, artemether was found to be no better than quinine (Murphy et al. 1996).

It has been reported in Thailand that artemether and artesunate were more effective than quinine or halofantrine at reducing rosette formation and cytoadherence (Udomsangpetch et al. 1996). Reduced sensitivity to quinine has been reported in Thailand (Karbwang et al. 1995, Udomsangpetch et al. 1996) and may account in part for the differences between rosetting sensitivity to the drug found in the Thai study and the study presented here. This study looks at five drugs not included in the Thai study. We measure a range of concentrations of each of seven drugs, while the Thai study centred on one concentration of four drugs. We have shown how rosetting is reduced when combining pairs of drugs. An additional difference between the two studies is that we used artemisinin and the Thai study used the artemisinin derivatives, artemether and artesunate. It would be interesting to measure rosette disruption by quinine and artemether or its derivatives and see if disruption correlates with efficacy of treatment in areas where quinine resistance has not been recorded. If such a correlation is found, it would suggest the potential use of agents to disrupt rosetting and cytoadherence in the treatment of severe malaria. Drugs disrupting rosetting and cytoadherence by altering parasite metabolism are likely to be more effective than antibodies against malarial surface antigens. We make this suggestion based on our studies where a pool of antimalarial antibodies, which disrupt cytoadherence (Goldring et al. 1992) and rosetting *in vitro* and were shown to bind to antigens on the infected erythrocytes surface (Bansil 1992) provided no detectable benefit when administered to

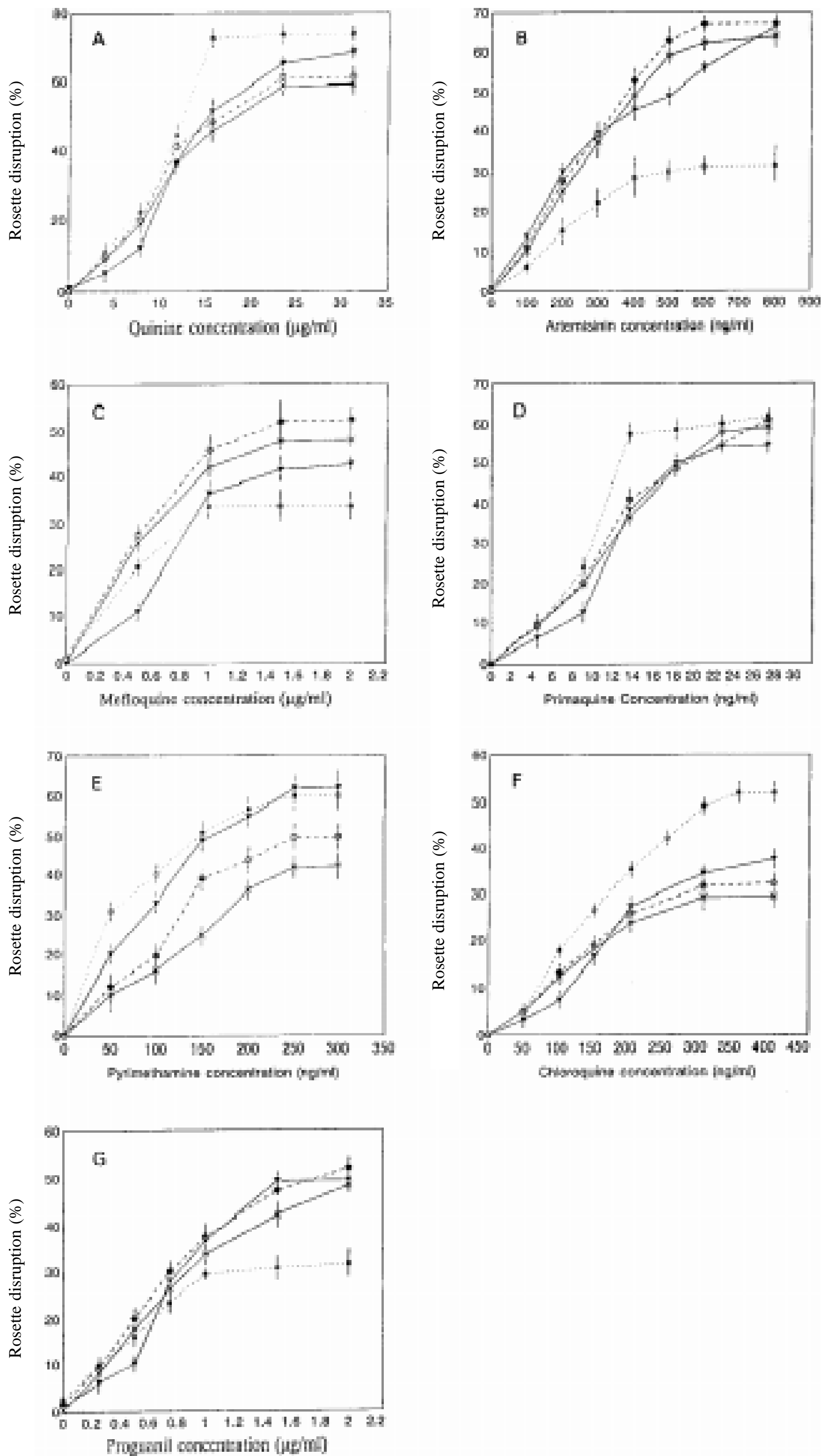

Fig. 1: the effect of quinine (A); artemisinin (B); mefloquine (C); primaquine (D); pyrimethamine (E); chloroquine (F) and proguanil (G) on the rosetting of *Plasmodium falciparum* infected erythrocytes. Each drug was incubated with parasite cultures, strain RSA 14( ), 15 ( ), 17 ( ) and FCR-3 ( ) at concentrations above and below therapeutic values and rosetting evaluated. The mean readings from three experiments performed in duplicate are shown with standard deviations - vertical bars.

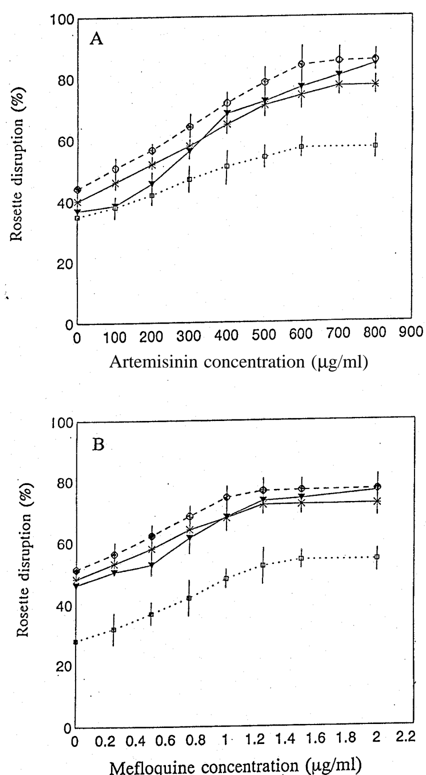

Fig. 2: incubating the therapeutic concentration of (a) artemisinin and varying the concentration of mefloquine, (b) mefloquine and varying the concentrations of artemisinin. Drugs were incubated with *Plasmodium falciparum* strains, RSA 14( ), 15 ( ), 17 ( ) and FCR-3 ( ) and rosetting determined. The mean readings from three experiments performed in duplicate are shown with standard deviations - vertical bars.

children with cerebral malaria (Taylor et al. 1992).

The different drug combinations also show varying effectiveness in disturbing rosettes. The combination of chloroquine and pyrimethamine are no longer recommended for prophylaxis and treatment of malaria in South Africa (Baker et al. 1993). In the rosette disruption assay, the combination of chloroquine and pyrimethamine was not significantly better at disrupting rosettes (approximately 35%, Table) than chloroquine alone (approximately 34% in Fig.1G). Webster (1990) reported that the combination of quinine with pyrimethamine has been used successfully to effect the suppressive cure of patients with multidrug-resistant malaria. In the present study this combination was better at disrupting rosettes than quinine alone. Combinations of mefloquine and sulfadoxine/pyrimethamine appear to be no better than mefloquine alone in the treatment of malaria (Sowunmi & Oduale 1995). In areas of mefloquine resistance, artemether combined with mefloquine appears to be useful (Karbwang et al. 1995, Bunnag et al. 1996). When artemisinin and mefloquine were combined in different concentrations we found the two drugs together reduced rosetting more effectively than each drug alone.

Anti-malarial drugs have been evaluated by measuring the number of parasites remaining or developing in the presence of the drugs. Determining rosettes is likely to be an indication of parasite death since as parasite metabolism slows down, protein synthesis decreases and so the number of rosetting ligands expressed by parasites also decreases. Ligands expressed on the surface of infected red cells are continuously turned over, for example cytoadherence ligands can be cleaved enzymatically and the ligands are re-expressed in a few hours (Hommel & Semoff 1988). Since all the drugs tested promoted reduced rosetting, we suggest that the assay is a method to measure drug sensitivity. It would be necessary to identify LD 50 values for any drug in a rosetting assay and compare values with those obtained in standard sensitivity assays, like the tritiated hypoxantine incorporation assay. Quinine is probably more effective than other drugs in our assay because it kills parasites quicker. The decrease in the number of uninfected red cells surrounding the parasitised cell will increase the vulnerability of the infected cell to the immune system and promote more rapid parasite clearance. Decreasing rosettes will also aid in reducing microvascular obstruction (Kaul et al. 1991). We think that our observations may begin to explain why one anti-malarial drug is more effective than another in treating patients with cerebral malaria, where rosetting and microvascular obstruction are linked to the severity of the disease (Carlson et al. 1990a).

TABLE

The effects of combinations of therapeutic concentrations of anti-malarial drugs on rosetting. The mean results of three experiments performed in duplicate are indicated ± standard deviations

Drug combination	% rosette disruption	Drug combination	% rosette disruption
Chloroquine/primaquine	55 ± 3.5	Pyrimethamine/primaquine	59.5 ± 4
Chloroquine/pyrimethamine	35 ± 3	Quinine/primaquine	81 ± 6
Chloroquine/quinine	64 ± 7	Quinine/pyrimethamine	80.5 ± 7

ACKNOWLEDGEMENTS

To Dr Theresa HT Coetzer for suggestions and comments.

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