

PLASMODIUM YOELII NIGERIENSIS : BIOLOGICAL MECHANISMS OF RESISTANCE TO CHLOROQUINE

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Summary :

The sensitivity to chloroquine according to the degree of synchronicity of *Plasmodium yoelii nigeriensis*, which is considered to be the most resistant of the rodent malaria strains, was studied. The infection was synchronised by means of a Percoll-glucose gradient which separates rings and young trophozoites from other stages. The mid term trophozoite, when it predominated in the blood at the time of treatment, was shown to be as sensitive to chloroquine as *Plasmodium vinckei petteri*. According to previous results indicating that part of the population of merozoites is latent and penetrates around midnight, the inoculations were timed in order to obtain a lower or higher degree of synchronisation. The infection appeared to be better synchronised if rings and young trophozoites, were inoculated at 06:00 hrs rather than at 15:00 hrs and consequently the efficacy of chloroquine was higher in the former than in the latter.

KEY WORDS : *Plasmodium yoelii nigeriensis*. Sporozoa. Rodent malaria. chloroquine. chemoresistance. merozoite latency. asynchronism.

ABBREVIATIONS : Ring (R); Young-trophozoite (YT); Mid-trophozoite (MT); Old-trophozoite (OT); Schizont (S); Intraperitoneal (IP); Intravenous (IV); Subcutaneous (SC); Multiplication rate (MR); Standard deviation (SD); Resistance index (RI); Synchronicity index (SI); Chloroquine (CQ).

Résumé : MÉCANISMES DE LA RÉSISTANCE À LA CHLOROQUINE CHEZ *PLASMODIUM YOELII NIGERIENSIS*

Le rapport entre asynchronicité et chimiorésistance est analysé chez *Plasmodium yoelii nigeriensis* qui, parmi les sous-espèces connues de *Plasmodiums* de rongeurs, est celle qui est spontanément la plus résistante à la chloroquine.

L'infection est synchronisée au moyen d'un gradient de Percoll - Glucose qui sépare les anneaux et les jeunes trophozoïtes des autres stades. Lorsque, avec ces infections rendues synchrones, le stade trophozoïte moyen est prédominant au moment du traitement, la chimiorésistance du *Plasmodium* disparaît.

Des travaux précédents ont montré qu'une partie des mérozoïtes libérés à la fin de la schizogonie reste latente et pénètre préférentiellement dans les hématies aux environs de minuit. Les stades jeunes synchronisés par le Percoll-glucose ont donc été inoculés à deux moments différents: soit à 6 heures pour que, à minuit, la schizogonie coïncide avec l'heure de pénétration préférentielle des mérozoïtes latents, soit à 15 heures moment où cette coïncidence n'existe plus. Il est constaté que dans le premier cas, l'infection est plus synchronique et plus sensible au traitement que dans le second cas.

MOTS CLES : *Plasmodium yoelii nigeriensis*. sporozoa. *Plasmodiums* de rongeurs. chloroquine. chimiorésistance latence des mérozoïtes. asynchronisme.

INTRODUCTION

Several murine malaria *Plasmodium* species show a spontaneous stable cross-resistance to various antimalarial treatments. *Plasmodium yoelii yoelii* 17X (Warhurst and Killick-Kendrick, 1967; Diggins and Gregory, 1969), *P. berghei* NK65 (Diggins *et al.*, 1969) and *P. yoelii nigeriensis* (Diggins *et al.*, 1969; Pande and Dutta, 1982) infections are to various extents innately resistant to chloroquine, quinine, mepacrine, amodiaquine and mefloquine. Beauté-Lafitte *et al.* (in press) showed with a prepatency test, that the minimum effective single dose of chloroquine active on *P. y. nigeriensis* was 50 mg/kg, i.e. 20 fold higher than that active against *P. vinckei petteri* (2.5 mg/kg). Interestingly, the biology of *P. y. nigeriensis* is also different : whe-

reas *P. v. petteri* infection is markedly synchronous with a schizogonic cycle of 24 hours, the parasitic development of *P. y. nigeriensis* in the blood of mice is naturally highly asynchronous, with a prolonged latency of a part of the population of merozoites which penetrate preferentially around midnight (Landau and Chabaud, 1980; Beauté-Lafitte *et al.*, 1993) and a short schizogonic cycle of 18 hours (Deharo *et al.*, 1994). A classification of 10 species, subspecies and strains of rodent *Plasmodia* according to their degree of sensitivity to chloroquine on one hand, and the extent of asynchronicity on the other hand, evidenced a correlation between asynchronicity and drug resistance (Beauté-Lafitte *et al.*, in press).

To confirm the role of asynchronicity in the drug resistance, it appeared necessary to study the dose-response effect of chloroquine in ordinary laboratory conditions, the potency of chloroquine on the sensitive stage of *P. y. nigeriensis*, the mid term trophozoite (MT) and finally, the variations of resistance according to the degree of synchronicity achieved by its artificial synchronization in the laboratory.

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MATERIALS AND METHODS

MICE

Outbred male Swiss mice (Charles-River, France) weighing 18-20 grams were used.

STRAINS

Experiments were performed with *P. y. nigeriensis* (NIG strain) from Nigeria. Published results (Cambie *et al.*, 1991) on *P. v. petteri* (279 BY strain) from the Central African Republic were used for comparisons with *P. y. nigeriensis*.

MICE INFECTIONS

Asynchronous infections: Mice were inoculated intraperitoneally (IP) with 0.2 ml of thawed blood from the same stock of aliquoted frozen infected blood. The stock comprised pooled blood taken from 10 mice with a parasitemia of 20 to 30% and frozen at -70°C with added glycerol, 5% final concentration. Since the onset of visible infection (parasitemias of 0.5 to 1%) parasite development was asynchronous.

Synchronized cycle : Synchronised infections were obtained by the method described by Deharo *et al.* (1994). Briefly, infected pooled blood from mice with parasitemias ranging from 15 to 25%, was centrifuged through a Percoll-glucose gradient which separates rings and young trophozoites from all other stages. Mice were inoculated intravenously (IV) with 0.5 ml of a suspension of RBC, 30% of which were infected by these young stages. With this procedure, the immediate resulting parasitemia in the receptor mice was 0.5 to 2%; the infection remained reasonably synchronous for the first two schizogonic cycles. The first schizogony occurred 15 hours post-inoculation and the following one 18 hours later.

SENSITIVITY TESTS

Non-synchronized infections : Mice were inoculated with frozen-thawed blood and were treated with a subcutaneous (SC) injection of a single dose of chloroquine base as soon as parasites were detected in the blood.

Synchronized infections : In man, peak plasma concentration of CQ is reached in less than 30 minutes after injection (White *et al.*, 1987). Shorter times (15'-20') were observed in mice (Cambie *et al.*, unpublished). Thus, treatment was administered SC on the patent infection at the time when MTs predominated, i.e., 9 hrs after the inoculation of Rs and YTs (from the Percoll-glucose gradient) or 12 hrs after the first schizogony.

FOLLOW UP OF PARASITEMIAS AND PARASITIC PATTERNS

Blood smears were prepared just prior to drug injection, 3 hrs and 12 hrs post-treatment, fixed in methanol and stained with Giemsa stain. Parasitemia was determined by counting at least 1500 cells, differentiating the various stages of parasite development to determine the parasitic pattern. The parasitic pattern is the percentage of each of the following parasite stages: R, YT, MT, OT, S, defined by Cambie *et al.* (1991) and Caillard *et al.* (1992). A synchronicity index (SI) was calculated as follows: SI = SD of the percentage of each stage / 50 (50 being the SD in a 100% synchronous infection). The index varies from 0 (asynchronicity) to 1 (synchronicity).

RESULTS

SPONTANEOUS RESISTANCE OF *P. Y. NIGERIENSIS* TO CHLOROQUINE

Two single doses of chloroquine were given to two batches of mice inoculated with frozen-thawed blood, 66 hours post-inoculation, when parasitemias were approximately 1%. One batch of 8 and one of 7 mice were treated, respectively, with 5 and 50 mg/kg. A third batch of 5 untreated mice was kept as control. Blood smears were performed at the time of treatment, 3 hrs post treatment and 12 hrs post treatment.

Just prior to drug injection, the infection was asynchronous, the parasitic pattern (not shown) displayed the presence of all stages. Three hours post-treatment, the parasitemia of control and treated mice increased slightly (Table I). Twelve hours post-treatment, the average rate of multiplication in control mice was 3.2 ± 0.26 , and slightly less in mice treated with 5 mg/kg (2.2 ± 0.4) and 50 mg/kg (1.8 ± 0.57). The multiplication rate was not significantly different between treated mice 5 mg/kg and treated mice 50 mg/kg (Test U, $P \leq 0.05$). The efficacy of the treatment was very low and independent of the dose.

SENSITIVITY OF MTs TO CHLOROQUINE

In order to study the sensitivity of MTs the strain was first synchronized with a Percoll-glucose gradient. Mice were inoculated at 13:00 with the gradient layer containing mostly Rs. A single dose of 5 mg/kg chloroquine was injected SC 9 hrs post-inoculation, at 22:00, when Rs had developed into MTs. This procedure was repeated twice (experiments 1 and 2). Blood smears were prepared just prior to treatment, 3

Treatment (mg/kg)	04:30 T. Time	07:30 T. + 3 hrs	16:30 T. + 12 hrs	Multiplication rate at T. + 12 hrs	
Parasitemia (%)				mean	
-	2.3	2.8	6.8	2.95	
-	2.7	3.1	8.8	3.3	
-	3.4	3.9	12.1	3.6	3.2 ± 0.26
-	1.6	1.6	5.3	3.3	
-	2.0	2.1	6.0	3	
5	2.4	2.5	6.2	2.6	
5	2.0	2.8	3.8	1.9	
5	0.8	1.4	1.8	2.3	
5	3.1	2.8	6.5	2.1	2.2 ± 0.4
5	3.1	4.3	6.1	1.97	
5	0.98	1.6	1.7	1.7	
5	3.1	3.6	6.2	2	
5	1.4	2.7	4.2	3	
50	2.1	3.1	4.7	2.2	
50	1.98	2.6	4	2	
50	2.1	3.1	4.7	2.2	
50	2.3	4.6	6.5	2.8	
50	3.0	3.7	3.9	1.3	1.8 ± 0.57
50	2.2	3.1	3.2	1.5	
50	2.2	2.5	2.8	1.3	
50	3.6	4.7	5.0	1.4	
50					

Table 1 – Effect of chloroquine on an asynchronous infection with *Plasmodium yoelii nigeriensis*.

T., time of treatment; T. +3 hrs, 3 hours post-treatment; T. + 12 hrs, 12 hours post-treatment.

hrs post-treatment when the transformation of MTs into OTs was observed, and 12 hrs post-treatment (6 hrs after the first schizogony), and the parasitic pattern and parasitemia were analysed. Control mice were observed in parallel.

Results are depicted in Table II. In all mice, infection developed synchronously. In the control mice, parasite stages developed as expected with *P. y. nigeriensis*: MTs developed into OTs within three hours; YT peak occurred at 10:00, six hours after the time of the predicted schizogony; the multiplication rate between 22:00 and 10:00 the next day was 2.6 (experiment 1) and 3.2 (experiment 2). In the treated mice, the transformation of MTs into OTs 3 hours post-treatment was diminished in comparison with that in the control mice, and the remaining MTs showed signs of degeneration: drug-induced vacuolisation of the cytoplasm and the characteristic pigment clumping. Twelve hours post-treatment the parasitemia stagnated, showing that schizogony did not occur. The YT stage expected at 10:00, i.e., 6 hrs post-schizogony, was almost completely obliterated and the predominant stage seen was altered OTs.

The activity of chloroquine (5 mg/kg) on the MT stage of *P.y.nigeriensis* appears to be comparable to that of the same dose on the MTs of *P. v. petteri*. Cambie *et al.* (1991) showed with *P. v. petteri* the

same morphological alterations, as well as an impairment of the transformation of MTs into OTs, resulting in an almost complete loss of schizogony and a decrease of parasitemia. However, due to the differences in the biology of the two species, the results are not exactly the same. The developmental cycle of *P. v. petteri* is 24 hours instead of 18 hours in *P. y. nigeriensis* and the transformation of MTs into OTs takes 6 hours instead of 3 hours respectively. In consequence, i) due to its rapid pharmacokinetics, the effect of chloroquine 3 hours or 6 hours post-injection is not absolutely equivalent; ii) damaged MTs may take more than 3 hours to be eliminated from the circulation and thus, be computed or not, according to the species, when evaluating the parasitemia and the parasitic pattern. In conclusion, despite these delineated differences, it appears that the MTs of *P. y. nigeriensis* and those of *P. v. petteri* are equally sensitive to chloroquine.

SENSITIVITY OF *P.Y. NIGERIENSIS* ACCORDING TO THE SYNCHRONICITY OF THE INFECTION

We have previously studied the development of the parasitemia of *P. y. nigeriensis* during the first schizogonic cycles following the synchronization by the Percoll-glucose gradient (Beauté-Lafitte *et al.*, 1993).

EXPERIMENT 1				EXPERIMENT 2			
Time	T. 22:00	T. + 3 hrs 01:00	T. + 12 hrs 10:00	Time	T. 22:00	T. + 3 hrs 01:00	T. + 12 hrs 10:00
Treated mice				Treated mice			
Par. %	1.4	1.3	1.7	Par. %	0.5	0.6	0.5
R	3	3	7	R	3	0	6
YT	31	5	<u>34</u>	YT	2	0	<u>12</u>
MT	<u>106</u>	60*	24	MT	<u>39</u>	38*	3
OT	0	<u>62</u>	58*	OT	7	<u>22</u>	18*
S	0	0	43	S	0	0	13
Par. %	2	1.4	1.2	Par. %	0.6	0.6	0.6
R	2	3	5	R	2	0	4
YT	20	6	<u>30</u>	YT	2	2	<u>11</u>
MT	<u>172</u>	90*	19	MT	<u>51</u>	28*	2
OT	6	<u>42</u>	32*	OT	5	<u>31</u>	19*
S	0	0	34	S	0	0	25
Control mouse				Control mouse			
Par. %	1.6	1.9	4.1	Par. %	0.5	0.7	1.6
R	22	2	49	R	6	4	14
YT	34	2	<u>238</u>	YT	4	4	<u>118</u>
MT	<u>104</u>	99	16	MT	<u>39</u>	15	11
OT	0	<u>86</u>	21	OT	2	<u>48</u>	2
S	0	2	86	S	0	2	14

Table II – Evolution of the parasitic pattern and the parasitemia of *Plasmodium yoelii nigeriensis* after the injection of a single dose of chloroquine 5 mg/kg at the time of mid-term trophozoites predominance.

* Altered parasites, T. time of treatment.

Underlined stages : the progression of parasite propagation can be followed by reading the parasitic stages in third row column 1 to column 2 in the fourth row and again in column 3 of the second row.

We showed that the parasitemia varied according to the time of inoculation and suggested that this phenomenon was related to the rate of penetration of latent merozoites which was mostly around midnight. Thus, when the time of inoculation was set so that the time of the first schizogony coincided with the optimal time of penetration of latent merozoites, i. e., around midnight, intraerythrocytic parasitemias increased greatly over a short period, thus reinforcing the synchronicity of the parasite. The protocol for our experiment is summarized in Fig.1. The inoculum comprised of Rs obtained through a Percoll-glucose gradient and diluted to 1/4 in saline. It was inoculated IV to batches of mice either at 06:00 or at 15:00, the first promoting a better synchronization than the second: the inoculation performed at 06:00 favours the synchronicity, the time of the first schizogony (21:00) allowing the simultaneous penetration of latent and rapid merozoites. To the contrary, when the inoculation is performed at 15:00, the time of the first schizogony (06:00) is well away from the preferential time of penetration of latent merozoites. The experiments were performed three times with two

batches of mice: one of control untreated mice and one treated SC with 50 mg/kg chloroquine. To evaluate the differences of degree of synchronicity between inoculations at 15:00 and at 06:00, we calculated, for each mouse, at the time of treatment, the synchronicity index (SI). To evaluate the treatment's efficacy we calculated the multiplication rate (MR) of each treated and control mice between the time of treatment and 12 hrs post treatment and the resistance index (RI = MR in treated groups / mean MR in control groups).

Results of the three experiments are gathered in Table III and correlation between RI and SI is in Figure 2.

Inoculations performed at 06:00 result in a more synchronous infection than that obtained with inoculation at 15:00, as judged by the SI values (Test U, $P < 0.05$). The relationship between the degree of chloroquine's efficacy and that of the parasites' synchronicity is shown (Figure 2): the more asynchronous the parasite (low SI) the more resistant (high RI) ($P < 0.01$ by Spearman's rank test).

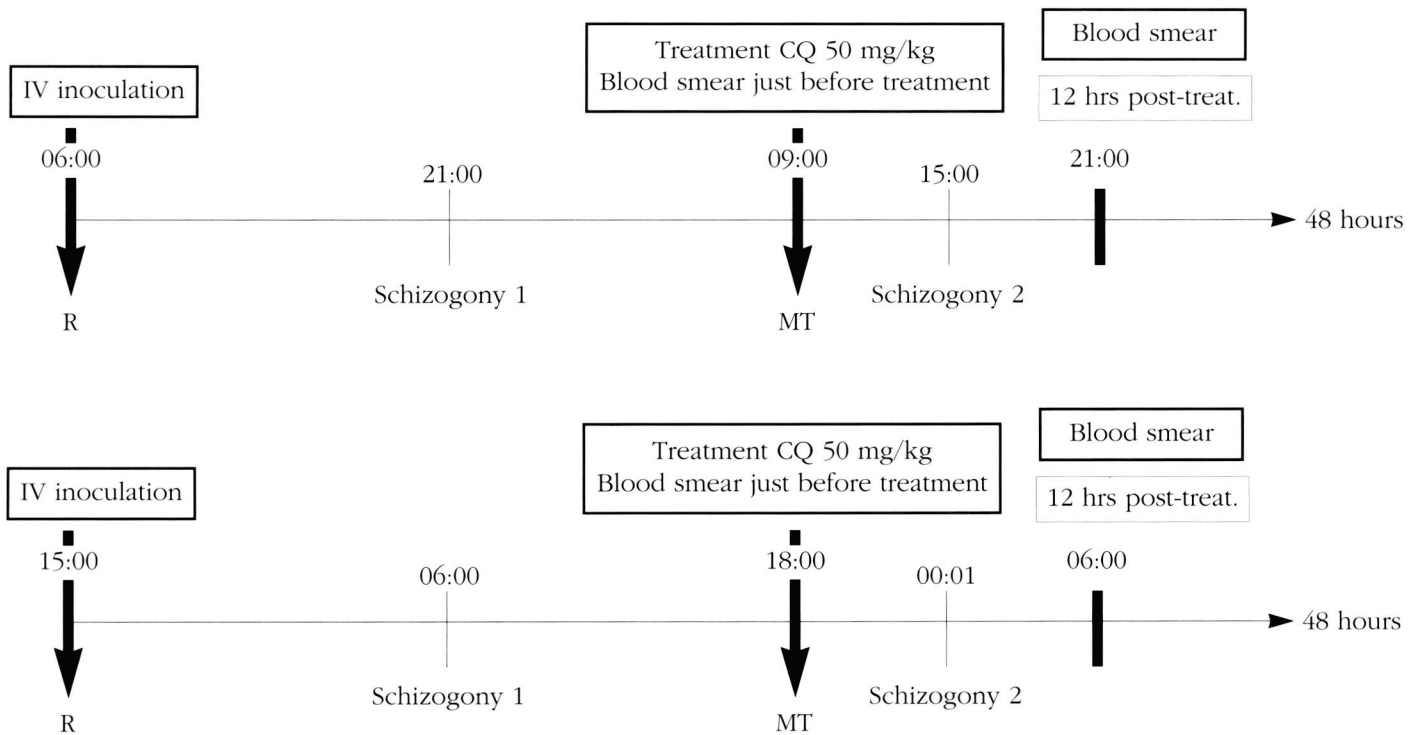


Figure 1. – Schematic protocol for studying the efficacy of chloroquine according to the degree of synchronisation i.e. the time of inoculation.

IV : intraveinuous; treat. : treatment.

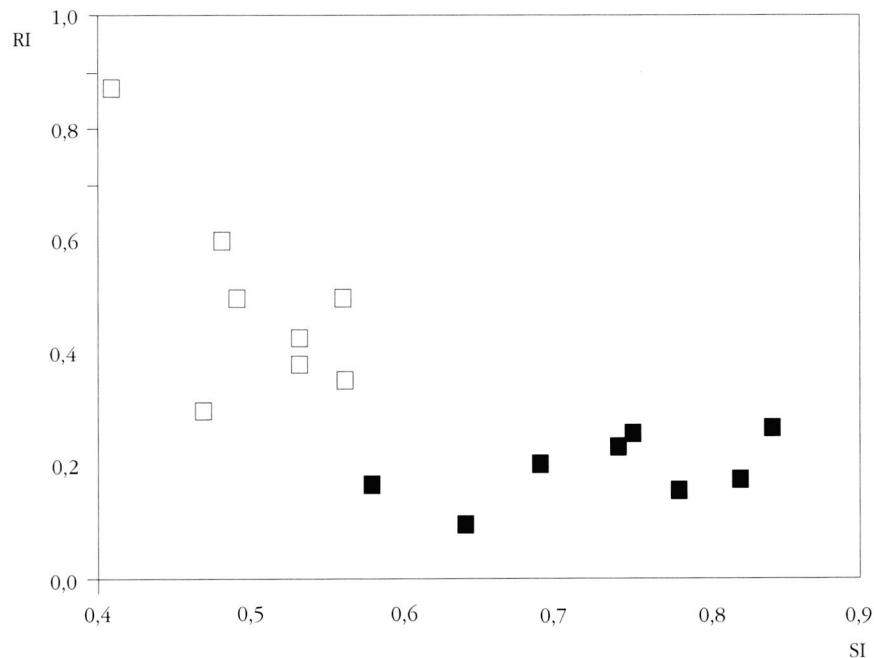


Figure 2. –Relationship between the degree of synchronicity (SI) and the sensitivity to 50 mg/kg chloroquine (RI) of *Plasmodium yoelii nigeriensis*.

SI : synchronicity index; RI : resistance index.

■ inoculation at 06:00; □ inoculation at 15:00.

CONTROL MICE – INOCULATION 15:00					CONTROL MICE – INOCULATION 06:00				
Parasitemia (%)		MR	SI		Parasitemia (%)		MR	SI	
T.	T.+12 hrs	mean	mean		T.	T.+12 hrs	mean	mean	
2.5	12		0.55		0.9	3.4		0.78	
1.8	7.5		0.61		0.2	0.6		0.65	
2.8	15.3		0.54		4.8	13.2		0.69	
2.4	5.3	4 ± 1.08	0.52		1.95	9.1	3.5 ± 0.6	0.59	
6.9	31		0.5		0.3	0.9		0.54	
5.3	19		0.5		7.9	28.5		0.73	
3.3	11		0.53		8.2	24.6		0.80	
–	–	–	–		2	7.5		0.68	

TREATED MICE – INOCULATION 15:00					TREATED MICE – INOCULATION 06:00							
Parasitemia (%)		MR	SI		RI	Parasitemia (%)		MR	SI		RI	
T.	T.+12 hrs		mean			T.	T.+12 hrs		mean			
5.3	18.6	3.5	0.41		0.87	2.3	1.9	0.83	0.74		0.24	
8.2	19.7	2.4	0.48		0.6	3	1.9	0.6	0.58		0.17	
5.4	6.3	1.2	0.47		0.3	3.9	1.4	0.36	0.64		0.1	
2.1	2.9	1.4	0.56		0.5 ± 0.05	0.35	8.5	5.4	0.64	0.82		0.73 ± 0.08
5.2	9	1.7	0.53		0.43	7.7	5.6	0.73	0.69		0.21	
5.9	11.7	1.98	0.56		0.5	7.2	6.9	0.96	0.84		0.27	
7.4	11.1	1.5	0.53		0.38	8.4	4.8	0.57	0.78		0.16	
5	10.2	2	0.49		0.5	7	6.3	0.9	0.75		0.26	

Table III – Activity of chloroquine according to the higher (inoculation at 06:00) or lower (inoculation at 15:00) degree of synchronisation of *Plasmodium yoelii nigeriensis* by means of a percoll-glucose gradient.
 T. : time of treatment; T. + 12 hrs : 12 hours post-treatment; MR : multiplication rate; SI : synchronicity index; RI : resistance index.

DISCUSSION

We have previously observed that the degree of sensitivity to chloroquine of different species, subspecies and even strains of the murine malaria parasites differed from one parasite to the other, and that there was a relationship between their sensitivity and their specific biology (Beauté-Lafitte *et al.*, in press). We have interpreted asynchronous infections as resulting from an extended latency of merozoites, a stage apparently insensitive to the drug (Cambie *et al.*, 1991).

The data presented in this work show that the particularly strong resistance of *P. y. nigeriensis* to chloroquine is not related to an innate resistance of the mid-term trophozoite, which is the sensitive stage to chloroquine of *P. v. petteri* (Cambie *et al.*, 1991), of *P. falciparum* (Landau *et al.*, 1992) and very likely of all the malaria parasites. The MTs of *P. y. nigeriensis* are shown to be normally sensitive to chloroquine. By choosing the proper timing for the inoculation of Rs and YTs, separated from the other stages by the Percoll-glucose gradient, various degrees of synchronization can be obtained. Using this controlled synchronization, we demonstrate that a relationship between the degree of synchronicity of the infection and its sensitivity to chloroquine exists.

It appears that simple biological factors like the selection of strains with latent merozoites and the consequent asynchronism may be responsible for drug resistance to chloroquine. This new hypothesis suggests new strategies for drug design and targeting. It becomes gradually obvious that merozoites, either *per se* or by the impairment of their penetration into RBCs, are a most suitable target for chemotherapy.

The rodent malaras so widely used for drug testing provide a large variety of models for chemotherapy studies. Work on chronobiology and chronotherapy can yield important information when a judicious choice is made in selecting the strain best adapted to screening or interpreting results on the mode of action of drugs.

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