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CHRONOTHERAPY OF MALARIA: IDENTIFICATION OF DRUG-SENSITIVE STAGE OF PARASITE AND TIMING OF DRUG DELIVERY FOR IMPROVED THERAPY

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SUMMARY

The cyclic nature of malarial fever in conjunction with the pharmacokinetic characteristics of antimalarial drugs, call for the conception of a chronotherapeutic approach for the treatment of the disease. An experimental murine malarial model was devised using the highly synchronous species *Plasmodium vinckei petteri* to test this rationale. Sub-curative doses of chloroquine were injected sub-cutaneously to mice either during the prepatent period or during patent infection. Inspection of the effet of drug applied at different stages of the parasitic cycle, revealed that medium size trophozoites (MT) were the most susceptible stage to chloroquine, while ring and young trophozoite stages were refractory to the drug. Chloroquine given during these latter stages, affected the parasites when they developed into the MT stage. Drug treatment during the MT stage phase-shifted the schizogonic cycle by 18 hours. Hence, treatment with two consecutive injections given 18 hours apart, *i. e.* timed to the overwhelming presence of the MT stage in the circulation, gave the best therapeutic results.

RÉSUMÉ : **Chronothérapie du paludisme : identification du stade sensible du parasite et horaires du traitement permettant d'améliorer son efficacité.**

Le caractère cyclique des accès de paludisme et les particularités pharmacocinétiques des médicaments antipaludiques permettent une approche chronothérapique du traitement de la maladie. Pour éprouver cette hypothèse, un modèle expérimental murin a été mis au point avec l'espèce hautement synchrone, *Plasmodium vinckei petteri.* Des doses sub-curatives de chloroquine sont injectées à des Souris, par voie sous-cutanée soit pendant la période pré-patente soit au cours des infections patentes. L'étude de l'effet du médicament administré sur différents stades du cycle érythrocytaire a montré que le trophozoïte moyen était le stade le plus sensible, alors que les anneaux et les trophozoïtes jeunes sont réfractaires. Lorsque la chloroquine est administrée au moment où les anneaux et les trophozoïtes jeunes sont présents, elles les fait disparaître lorsqu'ils atteignent le stade trophozoïte moyen. Le traitement au moment du stade trophozoïte moyen modifie le cycle schizogonique qui prend un retard de 18 heures. Il en résulte que les meilleurs résultats thérapeutiques sont obtenus avec deux injections consécutives de chloroquine lorsque celles-ci sont administrées à 18 heures d'intervalle et au moment où les trophozoïtes moyens sont majoritaires.

The pathology of malaria is characterized by its circadian rythmicity, that is evidenced by the correlation observed between the synchronous development of the parasite in the erythrocytes of its vertebrate host and the manifestation of the disease. During its eythrocytic cyclic propagation, the parasites undergoe major morphological, biochemical and physiological changes. It is therefore not surprizing that various drugs were found to exert differential effects on the distinct stages of parasite maturation (Yayon *et al.,* 1983; Dieckman and Jung, 1986; Geary *et al.,* 1989). These considerations, in conjunction with the pharmacokinetics of antimalarial drugs (Frisk-Holmberg *et al.,* 1984; Aderounmu *et al.,* 1987; White *et al.,* 1987), call for the contemplation of a chronotherapeutic approach to the treatment of malaria.

The goal of the present work is to propose a methodological approach for the *in vivo* evaluation of drug sensitivity of the various developmental stages of the parasite, and, consequently to time drug administration such that the peak drug level will be reached when the sensitive parasite stage is present in the circulation. It is presumed that such timing should increase the efficacity of drug treatment.

PRINCIPLES AND METHODOLOGY

Three experimental conditions were deemed prerequisite for testing the chronotherapeutic approach: 1) experiment-

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ing with a highly synchronous parasite species so as to be able to unequivocally identify the parasite stage most susceptible to the drug; 2) to use a drug concentration known to have sub-maximal parasitocidal activity and relatively short duration of active concentration in the blood; 3) to devise experimental protocols that would enable the identification of that parasite stages most susceptible to the drug, and that would consider possible alterations in the parasite's developmental rythm, for efficiently timing consecutive drug administrations in order to obtain maximal parasitocidal action.

The first condition was met using *Plasmodium vinckei petteri,* a highly synchronous species of murine malaria. Synchronicity was enhanced by rapid freezing and thawing of infected blood in 5 % glycerol solution that leaves only viable merozoites in the treated blood sample (Montalvo-Alvares *et al.,* 1988). The merozoites of this particular species invade erythrocytes readily after inoculation and no latent free parasites remain in the circulation (Cambie *et al.,* 1990).

The second condition was met using chloroquine at 5 mg/kg body weight injected subcutaneously at maximum time intervals of 6 hours.

The third condition was fulfilled using the following experimental protocols:

1 — Test 1: Outbred male Swiss mice (Iffa Credo) weighing approximately 20 g were inoculated intraperitoneally with freeze-thawed blood infected with *P. v. petteri* (279BY). Drug was administered once to different mice groups at 6 hours intervals starting immediately after inoculation. Thus, drug given to the first group (at time 0) would affect merozoites and the ring stage. Drug given 6 hours later to the second group of mice should sway young trophozoites. At 12 hours the drug would encounter medium size trophozoites, and drug given after 18 hours would imperil old trophozoites and schizonts. The development of parasitemia and the estimation of distribution of developmental stages (see below) was followed on consecutive days by microscopic inspection of Giemsa-stained thin blood smears, and compared to control mice (no drug). In a small proportion (± 10 %) of mice, parasite development was observed to be outstandingly different from that in the others. In order to obtain interpretable results, it was found necessary to discard those results that deviated by more than 3-fold from the group's mean.

2 — A second complementary test (Test 2), was performed 3-4 days post-inoculation, when parasitemia was sufficiently high to permit a precise study of the parasitic pattern (percentage of each stage present), but still far enough (at least 2-3 days) from crisis. Drug was administered in a single dose to groups of mice at different times of the cell cycle, when a definite developmental stage was overwhelmingly present in the blood. Following treatment, the parasitic pattern and overall parasitemia were evaluated every 6 hours, for 36-48 hours. Analysis of the evolution of these parameters allows the detection of the stage (or the stages) most affected by drug treatment, and to obtain an estimate of the duration of drug action given in a single dose. Underlying this test is the assumption that if one or more stages are eliminated by the drug, the periodicity of schizogony would be modified. Identification of the parasitic profile after drug administration is essential for detecting the time of reappearence of the most drug-susceptible stage.

Parasitic stages were classified as follows: Ring stage (R): less than a third of the erythrocyte (RBC) in size, displaying a large vacuole and a tiny ring of cytoplasm; Young trophozoite (YT): about a third of the RBC in size, having a large vacuole and little or no pigment; Medium size trophozoite (MT): between 1/3 and 2/3 of the RBC in size, smaller vacuole than in the previous stage and containing fine pigment particles; Old trophozoites (OT): 2/3 of the RBC in size, exhibiting small or no vacuole, dense cytoplasm, coarse and abundant pigment.

The parasitic pattern displays the proportions of the different developmental stages found in the sample blood at any given sampling time. Although each stage was considered for practical purposes to span 6 hours, the duration of the ring stage was in fact distinctly shorter (ca 3 h).

RESULTS

A — Effect of a single chloroquine injection during the prepatent period (1st test)

It has been shown previously (Landau *et al.,* 1990) that a single subcutaneous injection of 5 mg/kg of chloroquine diphosphate given during the 24 hours following inoculation prolonged the prepatent period of *P. v. petteri* but did not prevent subsequently the development of an otherwise apparently normal parasitemia. The prepatent period is defined as the time needed for parasitemia to reach 1 %. Using test 1 we found that following a single drug injection the longest prepatent periods were observed in those mice that were injected when they harbored medium size trophozoites *(Fig. 1).* In similar experiments performed with smaller doses of chloroquine (0.5 or 1 mg/kg), no extension of the prepatent period could be observed, implying that such doses are sub-liminal. Doses of 2.5 mg/kg prolonged the prepatent time by 2 days over that of controls, an extent similar so that obtained with 5 mg/kg.

B — Effect of a single chloroquine injection during patent infection (2nd test)

Since de infection of *P. v. petteri* is highly synchronous, the various stages of parasite development peak in the circulation at definite times of the day (Montalvo-Alvares

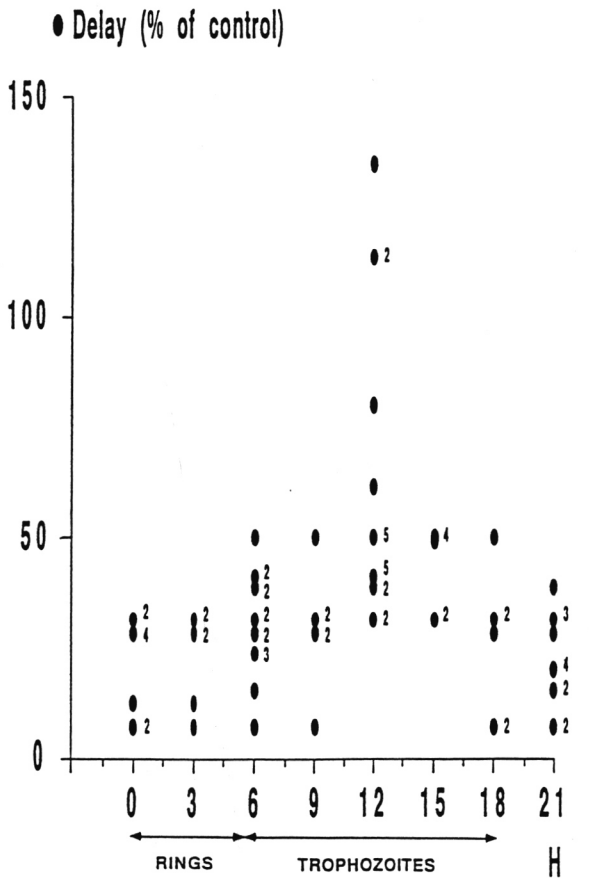

FIG. 1. — Chloroquine's activity is maximum 12 hours after inoculation of merozoites *i. e.* on actively growing trophozoites. This activity is estimated by counting the number of days while parasitaemias remain lower than 1 %. Each dot represents the delay of parasitaemia of a mouse as compared to the mean of untreated controls. The number besides some dots indicate the number of mice when several mice share the same values (Landau *et al.*, 1990).

et al., 1988). When inoculation is done at noon, schizogony occurs at 12 hours (noon), rings peak at 15 hours, young trophozoites at 18 hours, medium size trophozoites at 0 hour and old trophozoites at 6 hours. Five pairs of infected mice were used for each experiment. They were treated on days 5 to 7 post infection, each pair at a time of day that corresponds to peak appearence of a different stage, as defined above. The parasitic pattern was evaluated just prior to treatment *(Table I, 1st column),* and thereafter at 6 hours intervals. In experiments 1 and 2 however, the first blood smears were made 3 hours after drug administration. For the sake of comparison between experiments, results are presented as the product of the percentage of each stage and the percentage of parasitemia. The progression of parasite propagation can be followed in *Table I* by reading the figures in one row of column 1 to column 2 in the next row and again in column 3 of the next row. Once the last row is reached, the next pertinent value appears in the next column of the first row. For example, in experiment No. 1, stages deriving from rings in column 1 row 1, were underlined: YT appear in column 2 of row 2, MT derived from the same rings appear in column 3 of row 3, OT in column 4 of row 4 and R again in column 5 of row 1. The effect of drug treatment on a given stage can thus be evaluated by following the numbers in the columns from left to right, and downards. The effect of drug on OTs is evaluated by assessing the numbers appearing in the next column, top row. It should be noted that for *P. v. petteri* the expected multiplication rate is 3 (although the mean number of merozoites/schizont is 10), and since the ring stage lasts only 3 hours, the numbers in the top row essentially represent R + YT.

Results from 8 experiments (displayed in *Table I*) can be summarized as follows:

1 — chloroquine has an immediate inhibitory effect on MTs (exp. 5 and 6),

2 — chloroquine did not prevent growth of Rs and YTs but it became inhibitory to the parasites when they reached the MT stage (exp. 1 and 2),

3 — chloroquine did not prevent the development of OTs into schizonts and rings (exp. 7 and 8). In experiment 7, MTs were destroyed 18 hours after drug injection while in experiment 8, YTs appeared to be destroyed 6 hours post injection. Significantly. in the latter experiment MTs were also obliterated at 12 hours, *i. e.* 30 hours post injection.

4 — When a generation of MTs was annihilated by the drug, the parasite's schizogonic rythm (as determined from the parasitic pattern) was modified. The peak of each stage was phase-shifted (delayed) by 12 hours. In order to examplify this observation, it is seen in experiment 1, that between 18 hours and 0 hour, most MTs were killed. At 0 hour very few OTs could be seen and the percentage of YTs and MTs increased. At 6 hours, MTs which normally peak at 0 hour, were still very abundant. After that, MTs from 0 hour were also killed by the chloroquine still remaining in the blood, and the rythm showed a further 6 hours delay. Hence, development has been phase-shifted by 12 hours.

The stage dependency of drug sensitivity is graphically displayed by the displacement of the peak of rings and its height according to the stage present at the time of treatment *(Fig. 2)*. When drug is administered at the OT stage, there is no immediate effect, drug action is prolonged and the rythm is globally disturbed. Drug injection at the MT stage is immediately and highly effective, and cause a phase-shift of 18 hours. When drug is injected at the YT stage, the parasites continue to develop into the MT stage (6 h later) when they are destroyed. The cycle of those parasites that survived the drug, is phase-shifted by 6 hours. Treatment at the peak of the ring stage, has

TABLE I (Exp. 1-8). — *Single chloroquine injection. Treatment during patent infections (2nd test).*

EXP. 1 (CQ at 15H) : TARGET STAGE = R

Time (hrs)	15	18	0	6	12	18	0	6
Par. (%)	8	11,4	10,9	7,8	5,5	2,7	1,6	2,8
R	432	366	44	39	110	30	32	45
YT	304	638	286	172	132	57	46	118
MT	0	114	748	546	198	113	32	106
OT	64	23	22	23	110	70	50	11

STAGE DESTROYED: MT at 6H = DELAYED EFFECT

EXP. 2 (CQ at 15H) : TARGET STAGE = R

Time (hrs)	15	18	0	6	12	18	0	6	12
Par. (%)	8,2	11,4	9,7	6,4	4,5	2,3	3,7	4,6	4,3
R	303	285	150	19	8	60	130	23	17
YT	467	536	350	77	9	18	166	193	43
MT	0	536	480	435	135	60	26	221	228
OT	49	23	30	121	135	92	44	32	150

STAGE DESTROYED: MT at 0H = DELAYED EFFECT

EXP. 5 (CQ at 0H) : TARGET STAGE = MT

Time (hrs)	0	6	12	18	0	6	12
Par. (%)	1,95	0,8	0,24	0,14	0,18	0,24	0,38
R	2	0	2	1	1	0	1
YT	24	1	1	3	10	2	4
MT	138	30	5	2	5	16	10
OT	36	49	17	7	2	6	24

STAGE DESTROYED: MT at 6H = IMMEDIATE EFFECT

EXP. 6 (CQ at 0H) : TARGET STAGE = MT

Time (hrs)	0	6	12	18	0	6	12	18	0
Par. (%)	1,5	0,7	0,6	0,25	0,13	0,16	0,28	0,11	0,44
R	0	0	1	2	1	1	2	1	8
YT	13	5	5	2	5	5	5	3	28
MT	111	32	44	5	3	9	10	2	8
OT	25	32	10	17	3	2	13	5	2

STAGE DESTROYED: MT at 6H = IMMEDIATE EFFECT

EXP. 3 (CQ at 18H) : TARGET STAGE = YT

Time (hrs)	18	0	6	12	18	0
Par. (%)	1,1	1,1	0,7	0,1	0,1	0,4
R	18	0	1	1	4	12
YT	76	4	2	1	2	21
MT	16	78	43	1	1	4
OT	0	28	24	7	4	3

STAGE DESTROYED:MT at 12H = DELAYED EFFECT

EXP. 4 (CQ at 18H) : TARGET STAGE = YT

Time (hrs)	18	0	6	12	18	0	6
Par. (%)	0,5	0,3	0,2	0,2	0,07	0,07	0
R	7	0	0	2	1	1	0
YT	37	1	1	4	2	3	0
MT	5	18	10	5	2	3	0
OT	1	10	8	9	2	0	0

STAGE DESTROYED: OT at 18H = DELAYED EFFECT

EXP. 7 (CQ at 6H) : TARGET STAGE = OT

Time (hrs)	6	12	18	0	6
Par. (%)	0,7	1,6	1,3	0,7	0,48
R	12	18	6	2	0
YT	4	112	75	10	3
MT	2	18	46	48	22
OT	52	13	4	9	25

DELAYED EFFECT

EXP. 8 (CQ at 6H) : TARGET STAGE = OT

Time (hrs)	6	12	18	0	6	12	18	0	6
Par. (%)	0,34	0,46	0,13	0,23	0,24	0,07	0,08	0,26	0,15
R	1	19	1	1	1	1	3	4	0
YT	1	25	8	4	3	1	5	14	2
MT	2	2	0	16	11	2	0	6	5
OT	27	4	1	4	10	2	1	1	6

DELAYED EFFECT

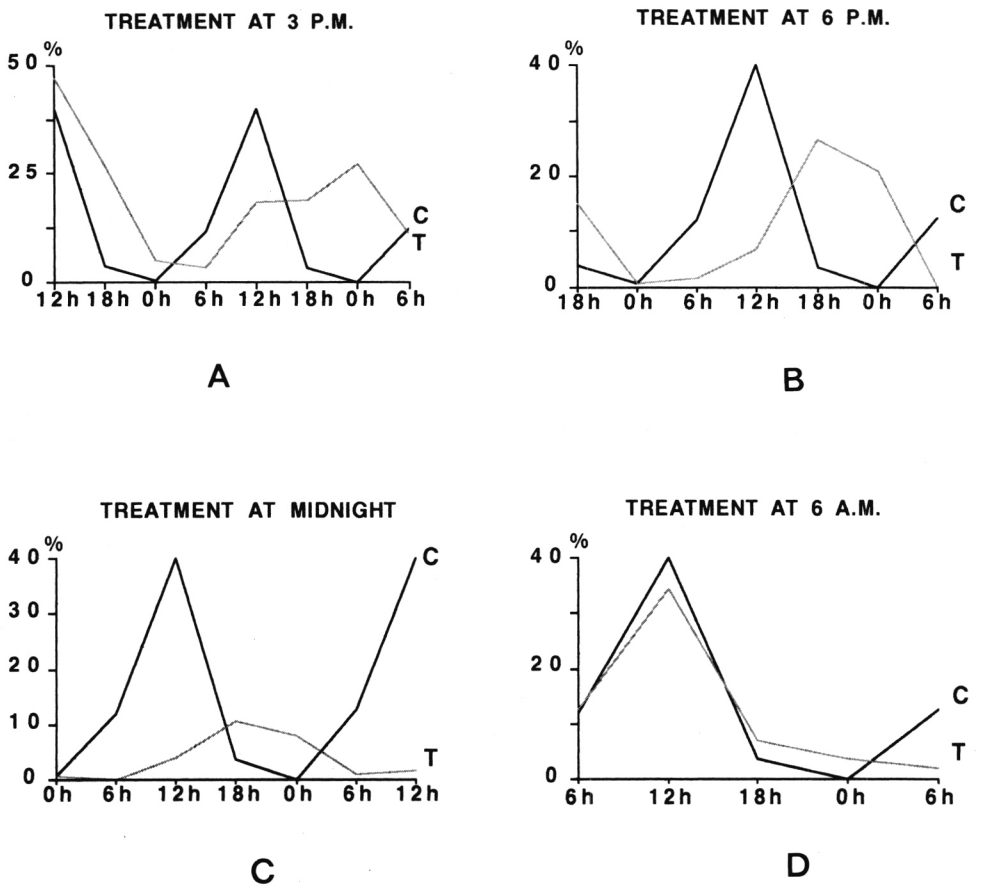

FIG. 2. — Modifications of the schizogonic rythm following a single chloroquine injection. T = percentage of rings in mice treated with chloroquine. C = percentage of rings in control mice. A: Target stage R, delay 12 hours. — B: Target stage YT, delay 6 hours. — C: Target stage MT, delay 6 hours + asynchronism. — D: Target stage OT, asynchronism.

no effect on parasitemia until 12 hours later, when the parasites reach the MT stage and the rythm of the surviving parasites is delayed by 12 hours.

5 — Although chloroquine concentration in the blood is known to peak within 10-15 minutes after injection, it rapidly distributes in the body tissues ($t_{1/2}$ of ca 50 mn, White *et al.*, 1987) and is eliminated from the body by excretion with a half-time of several days. The two latter processes lower the blood drug levels. It seems therefore that peak drug concentrations achieved after injection, are cytocidal mainly to MTs. Drug levels still prevailing after 18-30 hours, are apparently sufficient to affect the most susceptible MT stage.

C — Effect of two consecutive chloroquine injections

Since MTs appeared to be the stage most vulnerable to chloroquine and in consequence of drug treatment at this stage the schizogonic rythm was indubitably phase-shifted, a series of experiments were conducted to determine whether a judicious timing of two consecutive treatments could increase the efficacy of therapy.

In the first series of experiments the first injection was given on day 0 (day of inoculation) and the second injection on day 1, *i. e.* during the prepatent period. Furthermore, since the circadian rythm of *P. v. petteri* is independent from that of the host (Cambie *et al.*, 1990), the experiment was planned to address this issue: mice were divided into two groups: the first consisting of 7 batches of 3 mice, was inoculated at midnight. The second group consisted of 3 batches of 3-6 mice and was inoculated at noon. The first injection was given at different times post-inoculation to hit the parasites at different stages. The second injection was given at different time intervals, up to 26 hours. Each group included a control batch that received no drug.

Results depicted in *Table II* indicate that the effect of drug treatment on the length of the prepatent period depended predominantly on two parameters: the stage present during the first injection, and the time interval to the second injection. Thus, a short (3-6 h) time interval between injection, resulted in a prepatent period of 7-8 days (batches 2, 7, 10, 16). It seems as if the second injection had only a marginal effect in that it killed only an addi-

TABLE II (Exp. 9). — *Comparisons between prepatent periods in mice, after two chloroquine injections with different target stages.* R = rings, YT = young trophozoites, MT = medium size trophozoites, OT = old trophozoites, D = day.

BATCH	NB. MICE	TARGET STAGE		TIME OF TREATMENT		TIME INTERVAL	SCHIZOGONY	PREP. PERIOD
		1st Treat.	2nd Treat.	1st Treat.	2nd Treat.	Treat. 1-2 (Hrs)		(Days)
MICE INOCULATED AT 0H								
1	3	CONTROLS						3
2	3	MT	MT	D0 - 14H	D0 - 17H	3	-	8
3	3	R	MT-OT	D0 - 0H	DO - 22H	22	-	10
4	3	MT	YT	D0 -12H	D1 - 3H	15	+	12
5	3	MT	MT	D0 -12H	D1 - 6H	18	+	14
6	3	MT	OT	D0 -12H	D1 - 12H	24	+	9
7	3	R	YT	D0 - 3H	D0 - 6H	3	-	8
MICE INOCULATED AT 12H								
8	6	CONTROLS						4
9	3	S-R	YT	D1 - 12H	D2 - 0H	12	+	10
10	3	S-R	R	D1 - 12H	D1 - 15H	3	+	8
11	3	R	R	D0 - 15H	D1 - 17H	26	+	10
12	3	MT	YT	D1 - 0H	D1 - 15H	15	+	10
13	3	MT	MT	D1 - 0H	D1 - 18H	18	+	7,∞,∞
14	3	OT	OT	D1 - 6H	D2 - 6H	24	+	11
16	4	YT	MT	D0 - 18H	D1 - 0H	6	-	7
17	5	OT	OT	D1 - 9H	D2 - 9H	24	+	7

tional small fraction of parasites that escaped the first treatment. Further extension of the interval resulted in an increased prepatent period (batches 4, 9, 12). The highest efficacy was obtained when chloroquine was injected consecutively at 18 hours interval, hitting the parasite each time at the MT stage. In batch 5, the mean prepatent period was 14 days and in batch 13 two mice maintained a parasitemia lower than 0.05 % indefinitely, while the third had a prepatent period of 7 days. The latter diverging result is an exception that is unavoidable in this type of experiments (see *Principles and Methodology*). The apparent discrepancy seen when comparing batches 14 and 17, both given treatments at the OT stage (normally relatively insensitive), 24 hours apart, can be explained by the fact that the OTs in batch 14 were 3 hours younger than those of batch 17, and probably still relatively more sensitive to the drug.

To further accentuate the differential susceptibility of MTs and OTs, the experiments done on batches 13 and 14 were reproduced on a larger scale (10 animals/batch). Results of this experiment shown in *Table III* indicated a significant difference in the prepatent periods: treatment of MTs 18 hours apart resulted in a mean preparency period of 9.4 days, while treatment of OTs 24 hours apart in a mean of 6.5 days.

The results of two consecutive injections given during patent infection are shown in *Table IV*. They can be summarized as follows:

1 — Parasitemias decreased already after the first injection and further declined after the second one.

2 — A late recrudescence of low amplitude occurred after the second treatment; parasitaemias were usually lower than 2 %, only one mouse exhibiting a parasitemia of 11 %.

3 — Recovery from infection occurred much later than in control, untreated mice: 19 days average after inoculation and 11 days after the second treatment, as compared to 9 days in controls. However, in contrast to what was seen in untreated mice or in those given a single treatment, there was never a true crisis in mice given two consecutive injections.

DISCUSSION

Rodent malarias provide a wide variety of experimental models for the investigation on the fundamental and applied biology and pharmacology of malaria parasites.

The mechanisms whereby they regulate their biological rythm *vis-à-vis* the circadian rythm of the host, appear to vary in different species (Cambie *et al.*, 1990). Hence, the choice of the suitable species for the modelling of human malarias must take these notions in consideration. This reasoning applies specifically to the chronotherapeutic approach that seeks to find the optimal timing schedule for treating economically and efficiently an attack of malarial fever.

Generally speaking, chronotherapy deals with variations of drug absorption, distribution, metabolization and excretion during the circadian cycle of the patient. In the case of malaria, a number of other parameters must be taken

TABLE III (Exp. 10). — *Comparisons between prepatent periods in mice, after two chloroquine injections with different target stages.* MT = medium size trophozoites, OT = old trophozoites.

MICE	TIME OF TREATMENT		TARGET STAGE		INTERVAL (Hrs)	PREPATENCY
	1st treat.	2nd treat.	1st treat.	2nd treat.	1st-2nd Treat.	(Days)
1	D1-0h	D1-18h	MT	MT	18	10
2	D1-0h	D1-18h	MT	MT	18	9
3	D1-0h	D1-18h	MT	MT	18	9
4	D1-0h	D1-18h	MT	MT	18	10
5	D1-0h	D1-18h	MT	MT	18	10
6	D1-0h	D1-18h	MT	MT	18	9
7	D1-0h	D1-18h	MT	MT	18	9
8	D1-0h	D1-18h	MT	MT	18	-
9	D1-0h	D1-18h	MT	MT	18	9
10	D1-0h	D1-18h	MT	MT	18	11
Control						4
Control						4
Control						4
Control						4
11	D1-6h	D2-6H	OT	OT	24	7
12	D1-6h	D2-6H	OT	OT	24	7
13	D1-6h	D2-6H	OT	OT	24	7
14	D1-6h	D2-6H	OT	OT	24	6
15	D1-6h	D2-6H	OT	OT	24	7
16	D1-6h	D2-6H	OT	OT	24	7
17	D1-6h	D2-6H	OT	OT	24	7
18	D1-6h	D2-6H	OT	OT	24	7
19	D1-6h	D2-6H	OT	OT	24	7
20	D1-6h	D2-6H	OT	OT	24	6
Control						4
Control						4
Control						5

TABLE IV (Exp. 11). — *Effect of two chloroquine injections on the prepatent period; treatment beginning at days 5, 6 or 7.* R = rings, YT = young trophozoites, MT = medium size trophozoites, OT = old trophozoites.

MOUSE	TARGET STAGE		% PARASITAEMIA		INTERVAL 1st-2nd TREATMENT (Hrs)	RECOVERY Days after 2nd treatment	MAX. PARASITAEMIA AFTER 2ND TREATMENT	
	1st Treat.	2nd Treat.	1st Treat.	2nd Treat.			Day	%
1	R-YT	YT-MT	8	2.8	45	8	6	0.23
2	R-YT	MT-OT	8.2	4.27	39	10	6	0.35
3	YT	R-YT	1.1	0.4	30	10	6	1.71
4	YT	MT-OT	0.5	0	36	11	8	11
5	MT	OT	1.95	0.38	36	13	9	1.3
6	MT	MT-OT	1.5	0.49	54	12	5	0.03
7	OT	MT-OT	0.7	0.48	24	16	13	0.6
8	OT	MT-OT	0.34	0.15	48	9	4	0.34

into account, namely, the circadian rythm of the parasite, the parasitic target stage most sensitive to the drug, and modifications in the parasite's rythm resulting from drug treatment.

The stage of parasite development most susceptible to chloroquine has been already investigated in cultures of *P. falciparum*. While Yayon *et al.* (1983) found the trophozoite stage to be the most susceptible, Zhang *et al.* (1986) claimed that it was the ring stage. This discrepancy may have resulted from differences in the morphological definition of the stages, from the tightness of synchronization or from the variation in the experimental protocols. It seems however, that as shown in the present work for *P. v. petteri,* in *P. falciparum* also the trophozoites stage is more sensitive to chloroquine, in as much as this stage is also the most susceptible to a variety of chloroquine analogs that may be performing by the same mode of action (Geary *et al.*, 1986).

Pharmacokinetic studies on the levels of chloroquine in healthy animals and humans, indicate that either SC, IV

or IM injections, resulted in a very rapid increase of drug concentration in the blood, followed by a rapid ($t_{1/2}$ of few hours) decline to about 25 % of the maximal level due to distribution in body tissues, and a much slower decline ($t_{1/2}$ of many days) due to excretion (White *et al.*, 1987; Aderounmu *et al.*, 1987). The various rate constants are apparently independent of dose (Frisk-Holmberg *et al.*, 1984). Thus, when a minimal dose of drug is injected (*e. g.*, insufficient for radical cure), as done in the present work, it is not unlikely that the peak drug concentrations prevail for a period that is sufficient to kill the most sensitive stage.

These results and their analysis clearly substantiate the temporal aspect of drug action, both in terms of drug disposition by the host and with respect to its effect on target parasite stage, and underscore the rationale of the chronotherapeutic approach. The latter is lended further support from the cumulative effect of timed sequential drug treatments. Hitting the parasite in a timed schedule of drug treatment has been demonstrated in the present work to augment the efficacy of handling the disease. Is is also possible that additional benefits can be achieved by the chronotherapeutic approach since the double therapy used in the present work prevented the development of crisis. This may indicate that keeping parasitemia at low levels may allow the immune system to engage in host defence against the parasite.

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