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LATENCY OF *PLASMODIUM* MEROZOITES AND DRUG-RESISTANCE. A REVIEW.

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Summary :
The authors summarize the results of recent work evidencing the existence of latent merozoites during the course of the erythrocytic cycle of the rodent *Plasmodia*. These merozoites, unlike the majority of merozoites released at schizogony, do not penetrate immediately into the erythrocytes and remain latent for a variable length of time. The merozoites of each of the species or subspecies show marked peculiarities which are responsible for the characteristics of their cycle.
The presence of latent merozoites free in the blood, the asynchronous development, and the resistance to chloroquine, are three closely related factors. Knowing that the merozoite is so far drug resistant, and that latent merozoites can maintain the infection for any length of time, it appears important to take into account these purely biological data, when studying the drug resistance of the human *falciparum* malaria.

KEY WORDS : rodent *Plasmodium*, merozoite latency, asynchronicity, drug-resistance.

Résumé : LATENCE DES MEROZOITES ET CHIMIORÉSISTANCE DES *PLASMODIUMS*. DONNÉES RÉCENTES
La revue résume les résultats de nombreux travaux récents qui démontrent l'existence, au cours du cycle érythrocytaire des Plasmodiums de rongeurs, de mérozoïtes latents. Ceux-ci ne pénétrent pas dans une hématie dès qu'ils sont libérés mais restent latents un temps plus ou moins long. Les mérozoïtes de chaque espèce ou sous-espèce ont à ce point de vue des particularités très marquées et ce sont elles précisément qui expliquent les caractéristiques du cycle.
La présence de mérozoïtes libres dans le sang, la synchronie et la résistance à la chloroquine sont trois facteurs étroitement liés. Sachant que le mérozoïte est le stade qui résiste aux médicaments et sachant que les mérozoïtes latents peuvent entretenir longtemps l'infection, ces données purement biologiques paraissent importantes à considérer dans l'étude de la chimiorésistance du paludisme humain à P. falciparum.

MOTS CLES : *Plasmodiums de rongeurs, latence des mérozoïtes, asynchronie, chimiorésistance.*

Drug-resistance among malaria parasites is a particularly serious problem in tropical medicine and has generated a large number of experiments and publications. However this extensive litterature deals almost exclusively with biochemical or metabolical observations, and zoological or biological factors are not taken into consideration.

Work, during the past few years, has demonstrated indisputable correlations between the extent of the drug resistance and the biological pecularities of the various species or subspecies of the parasites of rodent malarias. These peculiarities concern, in particular, the more or less pronounced synchronicity or asynchronicity of the strains. Chronotherapy data (Cambie, Caillard, Beauté-Lafitte, Ginsburg, Chabaud and Landau, 1991; Landau, Chabaud, Cambie and Ginsburg, 1991; Caillard, Beauté-Lafitte, Chabaud and Landau, 1992) have shown that each drug acts generally on a precise parasitic stage and that the efficacy of the treatment depends on the proportion of the sensitive stage present at the time the drug reaches peak concentrations in the blood; consequently, asynchronous infections are much less sensitive than the synchronous ones.

Recent data led us to the conclusion that the rhythm and the synchronicity of strains are determined by the biological properties of the merozoites. It is generally thought that merozoites released by the schizonts invade immediately new erythrocytes or die. We think, on the contrary, that only part of the merozoites penetrate immediately (we called this population rapid merozoites = RapMs) while another population remains latent (=LatMs). The latter may disappear from the circulation (perhaps inside peripheral lymphatics or be sequestered inside deep capillaries) and reappear sooner or later, then they maintain the infection by reinvading new red blood cells (RBCs) (Cambie, Landau and Chabaud, 1990; Beauté-Lafitte, Chabaud, Altemayer-Caillard, Deharo, Gautret and Landau, 1993).

The ratio RapMs/LatMs varies from one species or subspecies of malaria parasites to the other. The higher the ratio, the more synchronous the strain. When we consider that the merozoites are resistant to

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most (or all) chemotherapeutic treatments it is easily conceivable that this latency and consequent late reinvasion, in association with the asynchronicity, may generate a drug resistance. These data may have relevance to the parasites of man since it is conceivable that, in the case of *P. falciparum*, strains with a relatively high proportion of latent merozoites could arise under drug selection pressure (Landau, Cambie and Chabaud, 1990).

It was generally thought that all merozoites penetrated the RBCs immediately after their release - a dogma, without experimental proof. However the opposite proposition i.e. some merozoites remain latent for variable length of time, is equally difficult to prove directly. This is why we tackled with this problem using different methods aimed at revealing the biological peculiarities of the merozoites.

I. – METHODS FOR STUDYING THE BIOLOGY OF MEROZOITES

Synchronization by freeze-thawing of infected blood

The injection into a host of RBCs in association with glycerol without re-establishing the osmotic pressure is known for its consequences on the cells which burst immediately. The intracellular parasites die and the infection develops only if merozoites free in the plasma survive the process. Montalvo-Alvarez, Landau, Baccam, Chabaud and Ginsburg (1988) showed that this was indeed the case with *P. v. petteri*, a highly synchronous subspecies with a 24 hours cycle; whatever the time of inoculation with frozen-thawed blood, schizogony occured 24 hours post-inoculation which corresponds to the time of development from merozoite to schizont. When fresh blood is passaged from mouse to mouse, the timing of the schizogony is the same in the donor and receptor mice.

Much information was obtained with mice inoculated with frozen-thawed blood :

a – the analysis of the parasitic pattern during the circadian cycle shows that if the rhythm of a synchronous strain is independent of the circadian rhythm of the host, the timing of the schizogony (and of all stages) depends on the time of inoculation (see *P. vinckei* section III). On the contrary, if the hosts' circadian rhythms are involved, schizogony will, in general, occur at a time not in accord with the inoculation time (see *P. chabaudi* section IV).

b – the analysis of the multiplication rate in relation to the circadian cycle of the host enabled us to estimate approximately the abundance of the RapMs (which determine an increase of the parasitaemia just after schizogony) and that of LatMs (responsible for a more progressive increase).

c – the passage of blood (after freezing) from donor to receptor mice enables the detection of LatMs. Based on the hypothesis that after infected blood has been frozen-thawed and inoculated, all intraerythrocytic stages are destroyed and the only live parasites are the free merozoites, the following experiments can be performed (Figure 1) :

A donor mouse is inoculated with frozen-thawed infected blood. RapMs penetrate immediately into RBCs while LatMs probably hide somewhere and reappear more or less rapidly in the circulation. If blood from the donor mouse is frozen before the first schizogony, all stages issued from RapMs which develop intracellularly are destroyed ; the only remaining live parasites are the LatMs. If the receptor mice are inoculated with blood taken from the donor mice and containing circulating LatMs, they develop an infection. Thus, this technique allows us to detect the presence (or the absence) of LatMs in the donor mouse (Cambie *et al.*, 1990; Beauté-Lafitte, Altemayer-Caillard, Gonnet-Gonzalés, Ramiaramanana, Chabaud and Landau, 1994a).

d – according to the above hypothesis, progressive dilutions of the frozen blood from the donor mouse allow us, after the inoculation to receptor mice, to evaluate the number of LatMs present at a given time, in the blood circulation of the donor mice (Landau *et al.*, 1990; Beauté-Lafitte et al. , 1993).

Inoculation of infected blood lysed in distilled water.

This method was employed by Deharo, Gautret and Landau (unpublished) to inoculate large numbers of infective merozoites and follow up their rate of penetration in RBCs.

Synchronisation by injecting schizonts maturing in cultures

This method was set up by Mons, Janse, Boorsma and Van der Kaay (1985). Schizonts of *P. berghei* in rat blood were allowed to mature *in vitro*. They did not burst in cultures. However, when injected to naive rats, merozoites were freed and initiated an infection which remained synchronous during 48 hours. The method allowed the authors to determine the duration of the erythrocytic cycle of the ANKA strain of *P. berghei* (22 hours).

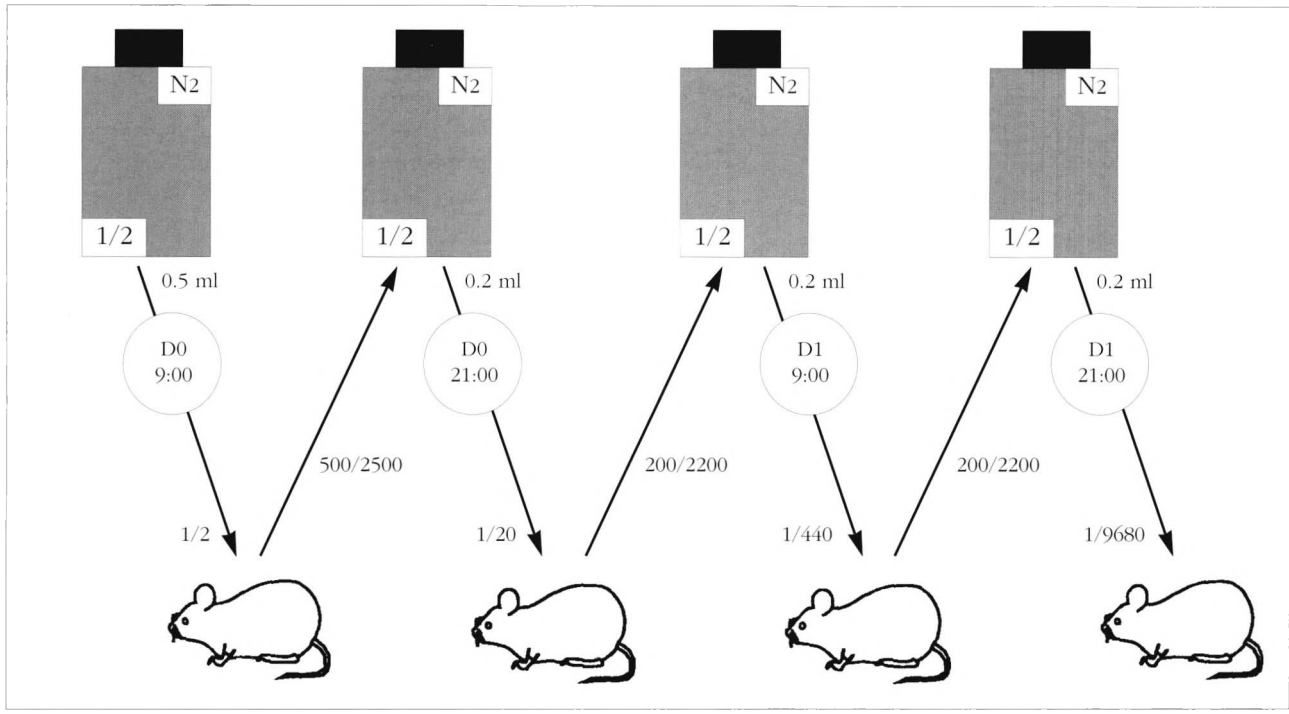

Figure 1. – Protocol for the estimation of the duration of the merozoites circulation.
The ratio above each mouse indicates the final dilution. It was calculated taking into account the total volume of blood in mice which was estimated to be 2000 µl, and the dilution to 0.5 of the frozen blood with glycerol. N2 = liquid nitrogen.

SYNCHRONISATION BY INOCULATION OF PARASITIZED BLOOD INTO A SENSITIZED HOST

Walter (1968) set up the following method "When blood containing *P. berghei* or *P. chabaudi* of the normal (asynchronous) course of infection from one host was inoculated into fresh animals of another susceptible species, which had been sensitized against the RBCs of the first host, hemolysis *in vivo* was followed by growth of a synchronous population, to which the young intraerythrocytic stages gave rise. This implies that only the merozoites and not other extracellular parasites are capable of invading erythrocytes and further development". According to this author, the duration of the erythrocytic cycle of the strain of *P. berghei* used was 17.5 to 18.8 hours.

DELAYED SUBINOCULATION OF FRESH BLOOD

This method was employed by Gautret, Deharo, Tahar, Chabaud and Landau 1994b, for studying the rhythm of *P. chabaudi*. Blood from a donor mouse with a normal day/night rhythm (schizogony at midnight) was taken in the morning, kept in a refrigerator at 4°C for several hours and then inoculated to receptor mice.

Three parameters were analysed every 6 hours during 12 days :

a – the parasitic pattern,
b – the Synchronicity Index,
- the Synchronicity Index (S.I.) calculated from data obtained by establishing the parasitic pattern is the ratio of the Standard Deviation (SD) of the percentage of each stage, at a given time, to the SD of a 100% synchronous infection. This index which varies from 0 (asynchronicity) to 1 (total synchronicity) is a good indicator of the degree of synchronicity.

c – the timing of the peaks of rings.
The timing and the height of the peak of rings were also considered because rings are formed three hours after the penetration of the merozoite, they are easy to characterize, of short duration, and very useful in determining the rhythm.

II. – CIRCULATION OF LATMS IN THE BLOOD

PENETRATION AT PREFERENTIAL HOURS.

Landau and Chabaud (1980) inoculated a frozen strain of *P. yoelii nigeriensis* to inbred mice at three different times of the same day; they observed that the prepatency increased progressively when the infective inoculations were performed at midnight, 16:00 and 09:00 (Figure 2). They thought at the time, that the infective merozoite could possibly only enter the RBC around midnight.
More recently, Beauté-Lafitte *et al.*, (1993) modified this hypothesis; they performed a series of experiments with *P. y. nigeriensis* synchronized by the percoll-glucose method and inoculated at different times

of the day. They came to the conclusion that merozoites entered RBCs principally on two occasions : just after the bursting of mature schizonts (RapMs) and around midnight (LatMs).

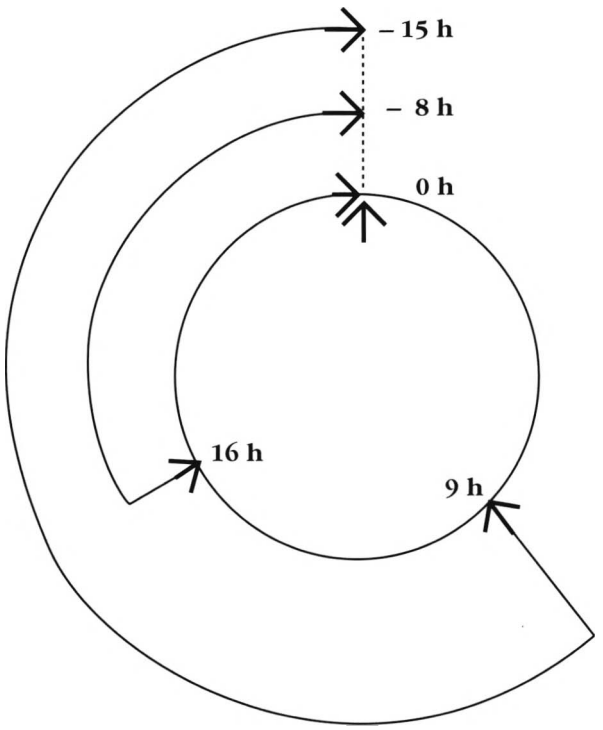

Figure 2. – Preferential penetration of merozoites of *P. y. nigeriensis* at midnight.
When merozoites were inoculated into white mice at midnight the infection became patent earlier than when merozoites were inoculated at 16:00 (8 hours delay) or at 09:00 (15 hours delay).

Detection of the LatMs in the circulation throughout the day ; specific characteristics of the strains.

The following experiments were devised to determine the duration of the merozoites latency (Cambie *et al.*, 1990).

Frozen and thawed blood infected with different species of *Plasmodium* was used to inoculate donor mice at 12:00. Blood of these donor mice was taken at 15:00, 17:00, 19:00, 00:01, 08:00, 12:00 and 18:00, frozen and thawed in order to destroy all the intracellular parasites and injected to naive mice (receptor mice). The abundance of latent merozoites (LatMs) in the inoculum was determined by the number of receptor mice of each batch becoming infected. The results showed essential differences between the three congeneric parasites of the same rodent host (Table I).

- *P. vinckei petteri* is a synchronous species, the timing of its schizogony depending on the time of inoculation. Merozoites were found in the circulation 3 hours post-inoculation ; they were scanty at 5 hours and could not be found 7 hours post-inoculation. They remained very scanty until schizogony time, 24 hours post-inoculation.

- *P. chabaudi chabaudi* is a less synchronous species and the timing of its schizogony depends on the circadian rhythm of the host, and occurs normally around midnight.

It was observed that at 15:00, 17:00 and 19:00, i.e. 3h, 5h and 7h post-inoculation merozoites which were at first scanty (? sequestered in the lymphatics) reappeared progressively, and that 12 h post-inoculation, i.e. at midnight, they became very numerous. After that they became again scanty until the schizogony and the onset of a new generation of merozoites.

- *P. yoelii yoelii* is asynchronous and the time at which schizogony predominates could not be ascertained. Merozoites were found to be numerous all day long in the circulation. All but one receptor mice became infected.

Thus, each parasite shows specific characteristics. We will explain in sections III, IV and V how the peculiarities described above may explain the differences between the cycles.

Subinoculation time	*P. y. yoelii*						*P. c. chabaudi*					*P. v. petteri*				
	1st experiment			2nd experiment												
15:00	–	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+
17:00	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–
19:00	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
00:01	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–
08:00	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–
12:00	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–
18:00	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–

Table I. – Demonstration of latency period before inoculated extracellular merozoites invade RBCs.
Donor mice were inoculated with frozen-thawed blood at 12:00. Blood was collected from them at the times shown, frozen and thawed and subinoculated into five or six recipient mice ; + : recipient became infected ; - : recipient did not become infected (modified from Cambie *et al.*, 1990).

ESTIMATION OF THE NUMBERS OF LATMS AND THEIR CIRCADIAN VARIATIONS BY EVALUATING THE LENGTH OF THE PREPATENT PERIOD.

It is a reasonable assumption that the prepatency is the longer when the parasite inoculum is the less and that, with the method of progressive dilutions, a correlation between the duration of the prepatent period and the number of merozoites inoculated can be established. Thus, the number of circulating merozoites in the blood of a donor mouse at a given moment, can be evaluated by freeze-thawing blood from this mouse, inoculating a given amount to receptor mice and determining the duration of the prepatent period. Results obtained with these methods could only be approximate (Beauté-Lafitte *et al*, 1993). We also know (see above, II a) that the hour of inoculation may modify the length of the prepatent period. Despite these difficulties it appeared that, whatever the time of inoculation, with *P. yoelii nigeriensis*, the preferential period for the penetration of merozoites was between 00:01 and 06:00. It appeared that amongst the merozoites freed at each schizogony, some penetrate immediately (RapMs) while others from the LatMs population only penetrate around midnight. According to our calculations, one merozoite (approximately) out of 10 remains latent at midnight, while at 15:00 only one out of a hundred is a LatMs.

III. – ERYTHROCYTIC CYCLE OF *P. VINCKEI*

The cycle in the blood of three subspecies of *P. vinckei* was investigated : *P. v. vinckei* from Zaire, *P. v. petteri* from the Central African Republic and *P. v. lentum* from the Congo Republic. The duration of the cycle was 24 hours for all the strains.

Experiments showed that when the inoculum was frozen-thawed blood, the schizogony of *P. v. petteri* and *P. v. vinckei* occured 24 hours post-inoculation whatever the hour of inoculation. This implies that almost all merozoites penetrate immediately into RBCs. The population of RapMs is largely predominant.

However the subspecies differ in several respects as revealed by the follow up of the parasitaemia and the parasitic pattern. The parasitic pattern can only be determined accurately when parasitaemias reach at least 0,5%; consequently the number of previous schizogonic cycles varies according to the virulence of the strain.

With *P. v. vinckei*, the strain was virulent and the parasitaemia was high enough on day 4 for the parasitic patterns to be established. In these conditions, the hour of the schizogony corresponded exactly to the hour of inoculation (Gautret, Deharo, Chabaud, Ginsburg and Landau, 1994a).

With *P. v. petteri*, when the infection was virulent and the parasitic pattern established on days 3 or 4 post inoculation, schizogony also occured at the hour of inoculation (Cambie *et al.*, 1990). However when the rise of parasitaemia was slower and the patterns were established from the 6th day onwards, the peaks of rings in most mice showed a delay of a few hours (Montalvo *et al.*, 1988); this discrepancy was, according to our interpretation, probably related to the number of previous schizogonic cycles in the donor animal: when these cycles were numerous, they tended to resume the natural rhythm they have in *Thamnomys rutilans* in the field (schizogony at 15:00) and this corresponds to a progressive reversion to the natural timing (Montalvo-Alvarez *et al.*, 1988; Landau and Chabaud, 1994).

With *P. v. lentum*, the parasitic patterns could only be determined late, after 9 successive schizogonies. The regulation which was then observed was surprising. The rhythm depended on both the time of inoculation and the circadian rhythm of the host; schizogony occured at 18:00 if the inoculum was injected at 06:00 or 12:00, and at 06:00 if injected at 18:00 or 00:01 (Gautret *et al.*, 1994a); this implies that merozoites were able to penetrate at two different times i.e. 06:00 or 18:00. To interpret this very particular rhythm, we propose the following hypothesis : gametocytes in the field are produced at two different hours, at the beginning and at the end of the night.

Knowing that many *Anopheles* species bite mainly at night dusk and dawn, it is possible that this periodicity represents an adaptation to the vectors biology. The gametocyte being short lived would be produced at two different times of the day in order to be mature and infective when the vectors feed.

IV. – ERYTHROCYTIC CYCLE OF *P. CHABAUDI*

The schizogonic cycle of the two known subspecies, *P. c. chabaudi* and *P. c. adami* was identical. When fresh blood was passaged from mouse to mouse, kept under light from 06:00 to 18:00, schizogony occured around midnight and the peak of rings around 03:00.

The strains although less synchronous than *P. vinckei petteri* were still markedly synchronous. It was shown by David, Hommel, Benichou, Eisen and Pereira da Silva (1978), that the timing of the schizogony of *P. chabaudi* varies when the circadian rhythm of the

infected mice is inverted. Cambie *et al.* (1990) confirmed this observation and showed that, contrary to what is observed with *P. v. petteri*, the timing of the schizogony is independent of the time of inoculation and is mainly governed by the circadian rhythm of the host.

Gautret *et al.* (1994b) showed that this specific periodicity is the result of an equilibrium established after several schizogonic cycles.

This species, as well as the others, produces LatMs and RapMs and the differences of periodicity can be explained with the notions expressed in section II :
if the inoculation is performed at midnight, the population of merozoites penetrating immediately after schizogony (RapMs) penetrates at midnight. The latent population (LatMs) penetrating preferentially also at midnight adds up to the previous one. The duration of the cycle being 24 hours, schizogony is established and maintained spontaneously around midnight.
It is therefore interesting to observe the rhythm when the time of the schizogony is artificially shifted (Gautret et *al.*,1994b). Mice were kept in normal daylight conditions; blood was taken at 10:00, from a donor mouse infected with *P. c. chabaudi*, when it contained mainly rings and young trophozoites, diluted in saline, and inoculated into receptor mice, either immediately or after a waiting period of 8 hours at + 4°C. When parasitaemias reached 1%, from D1 to D7, depending on the dilution, the parasitic patterns were studied every 6 hours over 2 or 3 days. The rhythm remained unmodified in mice inoculated at 10:00; in mice inoculated at 18:00 the infection was at first synchronous but the schizogony occured between 06:00 and 12:00 instead of midnight, as in the normal cycle. From day 4 to day 7 the infection became asynchronous. At day 10 the rhythm was resumed and the schizogony occured around midnight (Figure 3).
These phenomena can be explained if we consider that the RapMs undergo schizogony at an hour depending on the time of inoculation and the LatMs form an accessory "population" undergoing schizogony later and mainly at midnight. However RapMs and LatMs are not two truly distinct "populations" because wathever the type of the merozoite, it will give rise to schizonts that appear to produce the same proportion of RapMs and LatMs. During the successive cycles the progeny of merozoites penetrating at midnight increases progressively while that of RapMs formed at another time decreases and becomes almost extinct. Thus, one can expect : 1. a schizogony occuring at a time depending on the time

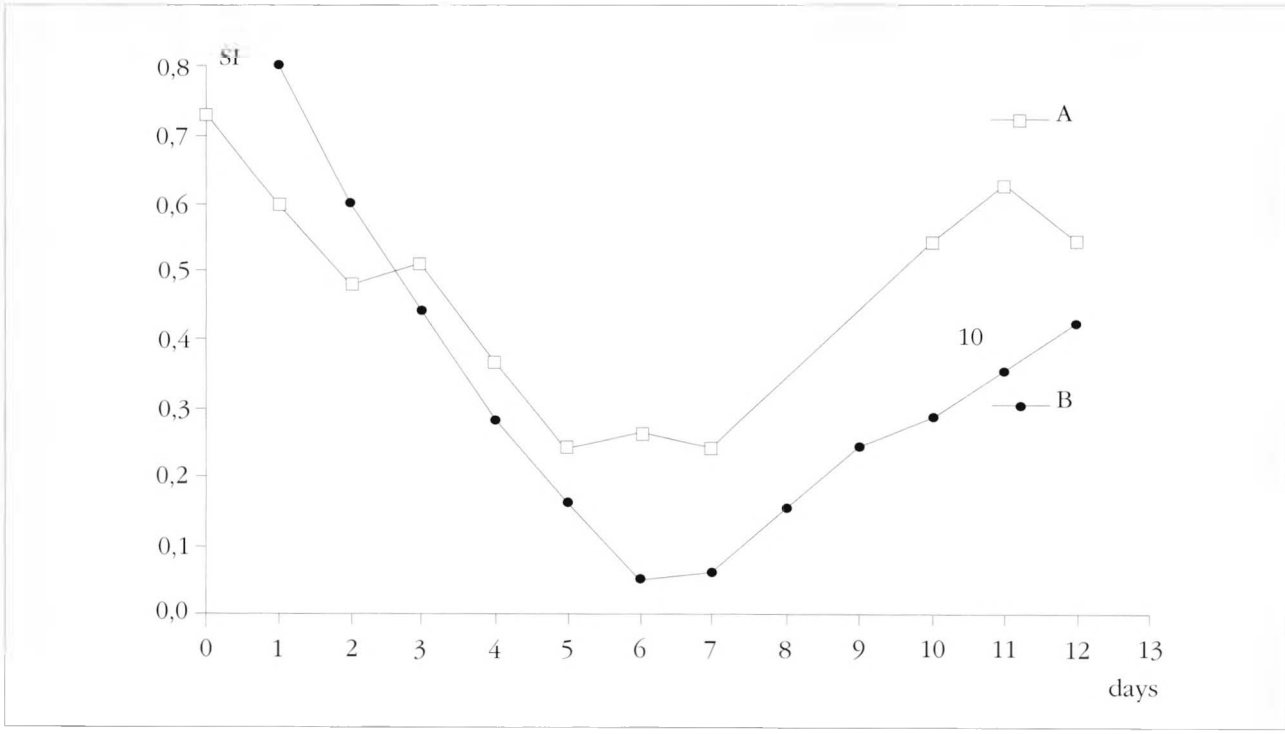

Figure 3. – Evolution of the Synchronicity Index (SI) of *Plasmodium c. chabaudi* during the 12 days following the inoculation at 18:00 of blood containing predominantly rings and young trophozoites (blood taken from the donor mouse at 12:00 and kept at 4°C for 6 hours) : curve A.
The mean multiplication rate of the strain being 4 and assuming a ratio of 90% of RapMs to 10% of LatMs at each schizogony, a theoretical calculation was performed. A curve was established with the ratio of the number of merozoites at 18:00 to the number of merozoites at 00:01 : curve B. The two curves are approximately parallel.

of inoculation during the first cycles, 2. a strong asynchronicity during the following cycles, 3. finally a synchronous infection again, with a different rhythm i. e. schizogony occuring at midnight.

V. – ERYTHROCYTIC CYCLE OF *P. YOELII*

All the subspecies of *P. yoelii* are asynchronous and impossible to synchronize for any length of time. However, infections synchronous during 2-3 cycles were obtained by Deharo, Gautret, Ginsburg, Chabaud and Landau, 1994; the youngest (rings and young trophozoites) erythrocytic stages of *P. yoelii nigeriensis* and *P. y. killicki*, were separated from the other stages using a discontinuous Percoll-glucose gradient. They were inoculated into mice and the subsequent infection remained synchronous for two generations only, after which the infection became progressively again asynchronous.

Experiments were difficult to follow over more than 3 cycles because parasitaemias became rapidly very high. However after inoculating mice with rings and young trophozoites of *P. y. nigeriensis* at 12:00 (=H0) and following the parasitic pattern every 3 hours for 3 days, the authors were able to follow a succession of 3 peaks of rings at 18 hours interval. At H4 the first peak (69%) represented the selection induced by the percoll-glucose. At H22, the peak became lower (55%) and decreased further to 40% at H40 (after the second schizogony), the infection having become at this time almost asynchronous. This loss of 15% at each cycle may indicate that a proportion of 15% LatMs against 85% RapMs which complete their cycle every 18 hours, could disorganize the synchronicity.

These results derive from the fundamental character differentiating *P. yoelii* from *P. chabaudi* – the duration of the schizogonic cycle – which is 24 hours for *P. chabaudi*, and 18 hours according to Deharo *et al.* (1994) or 17 hours according to Büngener (1985) for *P. yoelii*.

In principle, synchronisation of the latter cannot be achieved because, contrary to *P. chabaudi*, the progeny of the LatMs cannot accumulate progressively at midnight. Indeed, if we consider for example a synchronized inoculum of *P. y. nigeriensis* injected at a time set to obtain the first schizogony at 18:00, RapMs (which as indicated in II-c represent the dominant population) will, during the course of the successive schizogonies, appear at 18:00 (1st schizogony), after that at 12:00 (2nd schizogony), and then at 06:00 (3rd schizogony). It is only at the 4th schizogony that they will be released at midnight and penetrate synchronously with the LatMs. In all cases, merozoites at midnight (LatMs or LatMs+RapMs) will start a new series of desynchronizing schizogonies as indicated above. It is easy to understand that no synchronicity can be achieved by this species.

VI. – CORRELATIONS BETWEEN MEROZOITE LATENCY, ASYNCHRONICITY AND DRUG-RESISTANCE

MEROZOITE LATENCY AND DRUG-RESISTANCE

Landau *et al.*, 1990, on the basis of work on the sensitivity of the various parasitic stages to chloroquine, on the one hand, and on data concerning the latency of merozoites on the other hand, came to the conclusion that drug resistance and latency of merozoites are two closely related phenomena.

In order to confirm this hypothesis it appeared necessary to find a correlation between a. the sensitivity to chloroquine; b. the number of LatMs detected in the blood.

A systematic study of 10 species, sub-species or strains was performed by Beauté-Lafitte *et al.* (1994a). To test the sensitivity to chloroquine a prepatency test was used to determine the lowest dose inducing a significant delay of the prepatent period in comparison with controls. Doses varied from 2.5 to 50 mg/kg.

To detect the presence of merozoites in the circulation, merozoites were inoculated to a first mouse (with frozen-thawed blood); 12 hours later (before the occurence of any schizogony) blood from this mouse was taken, frozen and thawed, and inoculated to a second mouse, and this procedure was repeated with a third mouse etc... (Figure 1). Blood thus passaged was cleared of intra-cellular parasites and contained exclusively LatMs free in the plasma. Mice inoculated became infected or remained uninfected according to the degree of dilution (throughout the various passages to mice) 1/20, 1/440 and 1/9680.

When the strains studied were classified according to either their drug sensitivity or the decreasing number of LatMs the two series were almost identical (Figure 4).

The classification in descending order of drug sensitivity was as follows: 1) *P. chabaudi adami*; 2) *P. vinckei petteri*; 3) *P. v. vinckei* and *P. v. lentum*; 4) *P. c. chabaudi* and *P. berghei*, ANKA; 5) *P. berghei*, NK65; 6) *P. y. yoelii* and *P. y. killicki*; 7) *P. y. nigeriensis*. The classification in increasing order of number of LatMs was as follows : 1) *P. c. adami*; 2) *P. v. petteri* and *P. v. lentum*; 3) *P. c. chabaudi* 4) *P. berghei*, ANKA; 5) *P. berghei*, NK65; 6) *P. y. yoelii* and *P. y. killicki*; 7) *P. y. nigeriensis*.

Thus there was a high degree of agreement between the resistance to chloroquine and the prevalence of LatMs in the blood.

MEROZOITE LATENCY AND ASYNCHRONICITY

The synchronicity or asynchronicity of the strains is another factor unquestionably linked to the previous ones: the strains with the higher proportion of RapMs are the more synchronous and with the higher drug-sensitivity. Those with many LatMs penetrating at times differing from the RapMs (see chapter V, schizogony of *P. yoelii*) are asynchronous and relatively drug resistant.

ASYNCHRONICITY AND DRUG-RESISTANCE

With the percoll-glucose technique, *P. y. nigeriensis*, the most asynchronous and drug resistant of the malaria parasites, can be synchronized during two schizogonic cycles. The sensitivity to chloroquine of *P. y. nigeriensis* artificially synchronized, was studied by Beauté-Lafitte, Altemayer-Caillard, Chabaud and Landau, 1994b. These authors showed that the mid-term trophozoites of *P. y. nigeriensis* (a strain which is normally 20 times more resistant than *P. v. petteri*) was in fact as sensitive when synchronized, as the mid term trophozoite of *P. v. petteri*.

The same authors used another method to increase the synchronicity of *P. y. nigeriensis* i.e. by the inoculation of merozoites at midnight. The penetration of numerous LatMs coincided with that of the RapMs (see chapter II) and the infection was more synchronous than when the inoculation was performed at midday. The better synchronized infection was also the more drug sensitive.

CONCLUSIONS

The main characteristics of three species of rodent plasmodia are summarized in Table II. The interspecific variability in the numbers of latent merozoites appears to be an adequate explanation to the considerable differences observed between the schizogonic cycles of the rodent malaria species studied and all of the experimental results.

Each *Plasmodium* species produces two sorts of merozoites, RapMs penetrating into RBCs and beginning their development immediately after schizogony and LatMs penetrating only gradually with, however, a preference to either midnight or midday. The ratio RapMs/LatMs varies and characterizes the erythrocytic cycle of each species.

- With *P. v. vinckei* and *P. v petteri*, LatMs are few. The hour of inoculation of merozoites sets up the timing of

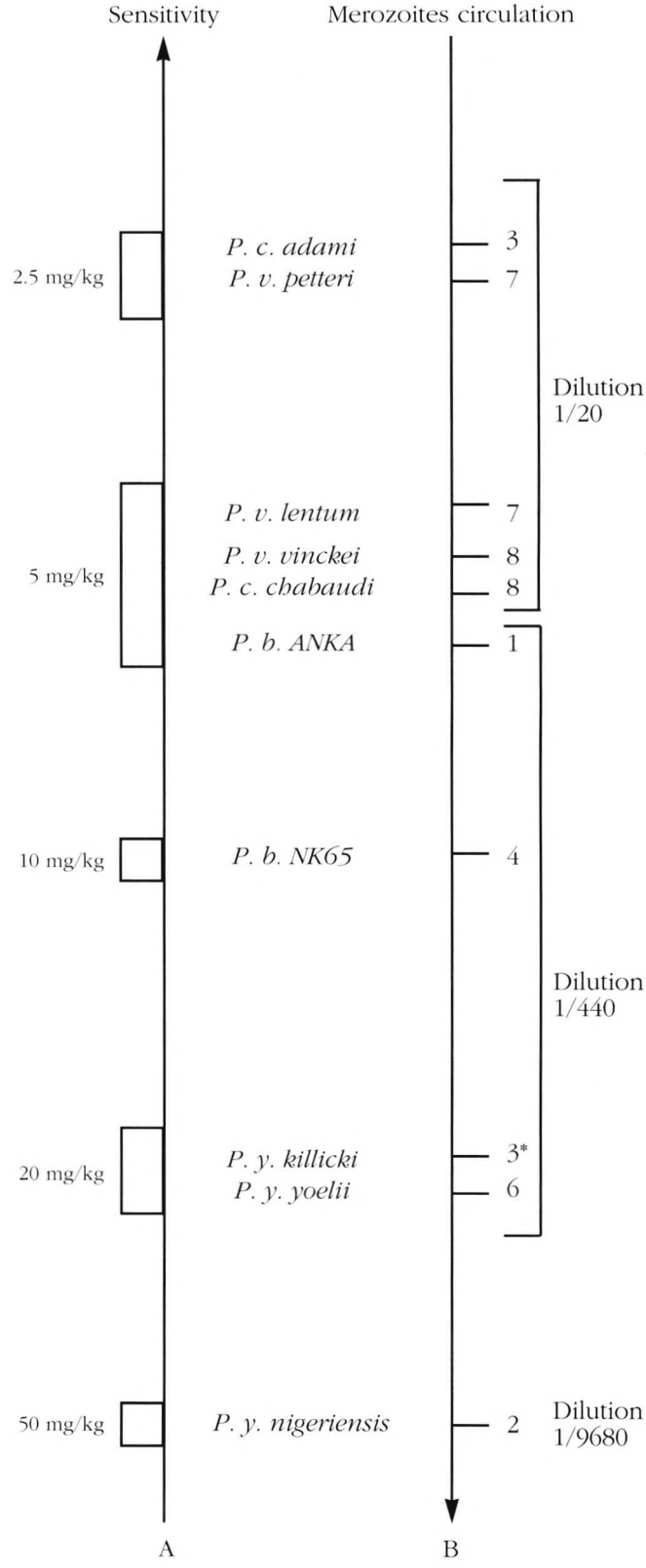

Figure 4. – Correlation between the sensitivity to chloroquine of 10 strains and the duration of their merozoite circulation. The figures along arrow B represent the maximum dilution determining an infection in the receptor mice and the number of mice out of a batch of 8 becoming infected (* only 5 mice per batch for *P. y. killicki*) (from Beauté-Lafitte *et al.*, 1994a)

	Cycle duration	Ratio LatM/RapM	Circulation of LatM	Penetration Time of LatM	Synchronicity	Drug Resistance
yoelii	18 H	+++	Permanent	00:01 = ++	+	+++
chabaudi	24 H	++	Maximum at 00:01	00:01 = +++	++	++
vinckei	24 H	+	Maximum at inoculation time	P. v. p.* 15:00 = +	+++	+

Table II. – Principal characteristics of the three species studied. (*the preferential time for the penetration of LatMs is 15:00 for *P. v. petteri*, but is 06:00 or 18:00 for *P. v. lentum*)

the schizogony. However after several schizogonic cycles, *P. v. petteri* tends to resume its natural rhythm with the schizogony around 15:00. With *P. v. lentum*, the cycle is particular, penetration occuring either at midday or at midnight, according to the hour of inoculation.

- With *P. chabaudi*, the proportion of LatMs is higher. The 24 hours duration of the cycle and the preferential penetration of LatMs at midnight enables one to understand how the schizogonic cycle is governed by the host circadian rhythm, and how if the schizogony is disturbed artificially, it re-establishes after seven or more schizogonic cycles.

- With *P. yoelii*, the proportion of LatMs is even more signifiant, as evidenced by the strong infectivity of the blood of mice (after freeze-thawing) all day long. However the schizogonic cycle lasting 18 hours, no synchronicity can be established and even if a synchronous infection is artificially set up, it becomes desynchronized after three cycles.

We assumed that merozoites were the only surviving stages after the freeze-thawing procedure. This cannot be proven directly and some colleagues prefer to consider that each species and each stage show different resistance capabilities to freeze-thawing procedures. Although the numerical data on the relative proportions of RapMs versus LatMs are indeed approximate, the bulk of the results obtained nonetheless shows links between the infectivity of the blood of infected mice (after freezing), the asynchronicity of the infection and the drug-resistance to chloroquine.

Büngener (1985) suggested that merozoites penetrating into reticulocytes develop into schizonts in 18 hours time, while their development is 30 hours inside normocytes. In our experiments with *P. y. nigeriensis*, at the first cycle after synchronisation by the percoll-glucose technique, almost all parasites occur inside the normocytes and their development time is 18 hours.

We believe that the reinfection by LatMs is one of the main obstacles to a successful treatment.
This should have considerable practical implications. Indeed merozoites seem to be resistant to all presently used antimalarials. The merozoite as well as the sporozoite are extracellular stages programmed to invade new cells and it is generally known in parasitology that these infective stages are also resistant stages.

If, as we suppose, merozoites of some strains of *P. falciparum* remain latent for a long period and escape treatment, such a latency which is a biological cause of drug-resistance in the rodent models could also play a role in the human malaria, independently from the biochemical or metabolical mechanisms put forward.

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