CHANGES IN SCHIZOGONY AND DRUG RESPONSE IN TWO LINES OF RODENT *PLASMODIUM, P. BERGHEI* NK 65 AND *P. BERGHEI* ANKA

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Summary :

White mice were infected with two strains, ANKA and NK 65, of *Plasmodium berghei*. The parasites were subjected to chloroquine pressure (60 mg/kg at each passage) during 20 passages. We then compared the behaviour of the strains as they acquired chemoresistance. The drug resistance was estimated by the 2 % delay time test (D2%), and the schizogonic rhythm by the synchronicity index (SI). Before drug pressure, the ANKA strain had a D2% of 4.34, and a SI of 0.2. This strain became highly drug resistant, but synchronicity increased: the D2% was 2.93, and the SI was 0.36 at the 20th passage. The NK 65 strain had an initial D2% of 4.12, and an SI of 0.2. The chemoresistance acquired during 20 passages was very irregular for this strain: after drug pressure, the D2% was 2.03 and the SI was 0.28. Drug pressure was then removed (for both strains), for 10 passages (no chloroquine). Resistance and synchronicity returned to their initial values. The two strains behaved very differently, in terms of their affinity for reticulocytes, and with chloroquine activity which favours an increase in SI because only merozoites are preserved.

KEY WORDS : *Plasmodium berghei*, NK 65 strain, ANKA strain, sensitivity, merozoites, chloroquine pressure.

Résumé : MODIFICATIONS DE LA SCHIZOGONIE ET DE LA RÉPONSE À LA CHLOROQUINE DE DEUX LIGNÉES DE *PLASMODIUM* DE RONGEURS, *P. BERGHEI* NK 65 ET *P. BERGHEI* ANKA

Des souris blanches ont été infectées par deux souches de Plasmodium berghei, *ANKA et NK 65. Les parasites ont été soumis à une pression de chloroquine (60 mg/kg à chaque passage) pendant 20 passages. Nous avons comparé le comportement biologique des deux souches pendant l'acquisition de la chimiorésistance. Le niveau de chimiorésistance est estimé par le test du délai à 2 % (D2%), et le rythme schizogonique par le test de l'Index de Synchronicité (IS). Avant la pression de chloroquine, la souche ANKA possède un D2% de 4.34 et un IS de 0.2. Elle devient très résistante au fil des passages, bien que sa synchronicité augmente, et au 20ème passage D2% = 2.93 et IS = 0.36. La souche NK 65 a un D2% initial de 4.12, et un IS de 0.2. La chimiorésistance acquise au cours des 20 passages est beaucoup plus irrégulière que celle de* P.b. *ANKA, et au 20ème passage, D2% = 2.03 et IS = 0.28. Par la suite, la pression médicamenteuse est supprimée et après 10 passages sans chloroquine, on observe un retour aux valeurs initiales de résistance et de synchronicité.* P.b. *ANKA et* P.b. *NK 65 montrent un comportement biologique très différent, en raison de leur affinité distincte pour les réticulocytes, et de l'action de la chloroquine, qui favorise une augmentation de la synchronicité en détruisant les stades parasitaires autres que les mérozoïtes.*

MOTS CLÉS : Plasmodium berghei, *souche ANKA, souche NK 65, sensibilité, mérozoïtes, pression de chloroquine.*

INTRODUCTION

The resistance of *Plasmodium* to chloroquine is now a major public health problem, because it concerns almost all countries where people are infected. But chloroquine is still widely used because it is cheap and has practically no side effects. People living in continuous transmission areas are simultaneously infected by several strains or species of *Plasmodium*. This makes analysis of the *in vivo* response of *Plasmodium* to drug treatment considerably more complicated. We have therefore developed a murine experimental model so as to obtain evidence of differences in the behaviour of two strains of the same plasmodial species, that both infect the same host in identical conditions. We tested two strains of the African Muridae (*Thamnomys rutilans*) *Plasmodium* for the induction of chemoresistance.

MATERIALS AND METHODS

DEFINITIONS (F.E.G. COX, 1988)

Primary isolate: a population of parasites in an experimental animal after the injection of parasites from a naturally infected host.

Strain: population of parasites derived from characterized isolates or characterized after isolation.

Line: this term should be applied to any particular derivation from a named strain, for example, one that has

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become particularly virulent or avirulent or has been adapted to an unusual host.

Biological materials

• Rodents

Female Swiss mice (18-20 g, Janvier, France) were used. They were kept under a 06:00/18:00 day/night rhythm.

• Parasites

- *Plasmodium berghei* NK 65 (NK for New-York/Katanga) was isolated from the invertebrate vector *Anopheles dureni millecampsi* captured in Katanga (near Lumbumbashi) in 1965.
- *Plasmodium berghei* ANKA (ANKA for Anvers/Kasapa) was isolated from the same host in Kasapa (Katanga), not far from the same site (WERY, personal communication).

• Chloroquine

The treated mice were given a single subcutaneous (s.c.) injection of 60 mg/kg-chloroquine diphosphate (SIGMA® CHIMIE).

General methods

• Definition of erythrocytic stages (Cambie *et al.*, 1991)

Ring (R): the smallest intracellular stage observed following invasion of erythrocytes by the merozoites.
Young trophozoite (YT): has a larger vacuole, a more developed cytoplasm, an irregular contour and very faint (if any) pigmentation.
Midterm trophozoite (MT): occupies one-third to one-half of the host erythrocyte volume and has a larger nucleus, relatively more abundant cytoplasm, a smaller vacuole, and a few pigment granules.
Old trophozoite: occupies one-half to almost the entire volume of the RBC and has a densely staining cytoplasm, a small vacuole and profuse pigmentation.
Schizont (S): a multinucleate form, counted with OTs.

• Follow-up of infections

- Blood parasite concentrations

Blood parasite concentrations (parasites per 100 RBC) were estimated by counting in 2,000 red blood cells in methanol fixed, Giemsa-stained thin films of tail blood.

- Parasite pattern

The parasite pattern was obtained by counting the percentage of each parasite stage (R, YT, MT, OT/S) and recorded according to the classification of blood stages proposed by Cambie *et al.* (1991).

• Resistance evaluation

- Degree of resistance (2 % delay time)

The degree of resistance was evaluated by the "2 % delay time test" (Warhurst, 1966), which is the difference between the number of days needed for the treated and control mice to reach a blood parasite concentration of 2 %. The shorter the time, the higher the resistance.

- Synchronicity index (SI)

The index (Beauté-Lafitte *et al.*, 1994 a) was calculated as follows:

$$SI = SD \text{ of the percentage of each stage}/50$$

where SD is the standard deviation.
The number of erythrocyte stages is 4: R, YT, MT, OT/S.
50: standard deviation of a 100 % synchronous infection when four stages are considered.
The index varies from 0 (asynchronicity) to one (total synchronicity). Thus, the index of a sample containing 100 % mid-term trophozoites is one. But a sample of the same infection taken three hours later may show 50 % mid-term trophozoites, 50 % old trophozoites and an index of 0.5. We therefore considered only the mean indices calculated from parasite patterns evaluated at six hours intervals over 48 h. The parasite patterns were evaluated in mice with blood parasite concentration of 0.1 % to 10 %, a substantial time before the crisis. The indices are usually identical in each mouse of a given batch under these conditions and are thus, reliable.

Experimental infection of mice

• Freezing and thawing procedures

Aliquots were prepared with blood of mice having a parasite concentration of approximately 20 %. Blood was collected by cardiac puncture under general anaesthesia. The stock mixture contained infected blood together with an equal volume of 10 % glycerol in saline and frozen immediately at – 70° C or – 196° C. The blood was then rapidly thawed, causing the RBC to burst, so destroying the intraerythrocytic stages; only the merozoites were preserved.

• Mouse infections

Mice were inoculated intraperitoneally (i.p) with 0.2 ml of thawed blood from the same stock of aliquoted frozen infected blood. The mice used for each new passage were given at noon 10^6 parasites in 0.2 ml saline. Mice were divided into two batches:
a) Treated mice: five mice were given 0.2 ml blood containing 10^6 parasites intraperitoneally (i.p). They were given a single subcutaneous (s .c) injection of 60-mg/kg chloroquine at the time of parasite inoculation.
b) Control mice: five mice were given 0.2 ml blood containing 10^6 parasites intraperitoneally (i.p).

Experiments

Drug pressure : experiments were performed simultaneously on *Plasmodium berghei* NK 65 and *Plasmo-*

dium berghei ANKA using batches of treated and control mice. Blood smears were examined daily until the parasite concentration was over 2 %. The parasite was then passaged from one of the treated mice into two new batches: one batch served as a control and the mice in the other batch were treated as described above. Twenty passages were performed, (usually about noon so as to target the most sensitive stage, the mid-term trophozoite, but because of the rapidly growing asynchronicity this precaution was not completely effective) in order to establish continuous drug pressure. The donor mouse was (in the treated batch) the one that took the longest time to reach a 2 % parasites, and was thus the mouse infected by the most resistant parasites.

The 2 % delay time (D2%) of the starting ANKA and NK 65 lines was estimated and used as a reference point (= passage 0). The 2 % delay time (D2%) was then calculated for each passage. Parasite patterns were calculated every six hours for 48 h in the 12th and 20th passages to determine the synchronicity indexes (SI) of the treated and control batch. The D2% was used to establish the tendency curve for the NK 65 and ANKA strains during the 20 passages, and so reveal differences in the behaviour of the two strains.

RESULTS

EVOLUTION OF D2% OF *PLASMODIUM BERGHEI* ANKA AND *PLASMODIUM BERGHEI* NK 65 (Fig. 1)

The D2% before any chloroquine pressure were 4.34 days for ANKA and 4.12 days for NK 65. These points served as references for the subsequent experiment. Let us remind that the D2% is greater when a *Plasmodium* strain is spontaneously resistant to chloroquine.

- *Plasmodium berghei* ANKA

D2% dropped from 4.34 to 2.93 between the passage 0 and the passage 20, indicating a sharp decrease in sensitivity to chloroquine. This change in D2% occurred in two phases (see Fig. 1 and Fig. 2: tendency curve of the D2% during the 20 passages). In the first, from passage 0 to passage 13, D2% decreased regularly from 4.34 to 1.85. The gradient of the corresponding tendency curve was – 0.21, and the average D2% was 3.31. In the second, from passage 14 to passage 20, D2% dropped from 3.64 to 2.93 days, with an average of 3.19. The gradient of this tendency curve was – 0.03, indicating the stabilisation to a plateau of D2%.

Values of D2% according to the passages

Passages	ANKA	NK 65
0	4,34	4,12
1	4,11	4,18
2	4,31	4,91
3	3,94	4,10
4	5,32	5,05
5	3,46	4,30
6	3,20	3,58
7	3,13	2,02
8	2,74	4,18
9	2,92	4,37
10	2,15	3,58
11	2,70	2,53
12	2,12	0,98
13	1,85	2,78
14	3,64	3,46
15	2,69	3,84
16	3,13	3,30
17	3,32	2,90
18	3,53	3,37
19	3,07	2,96
20	2,93	2,03
21	4,64	3,98

passage 0 = initial strain

passage 21 = 10th passage without chloroquine

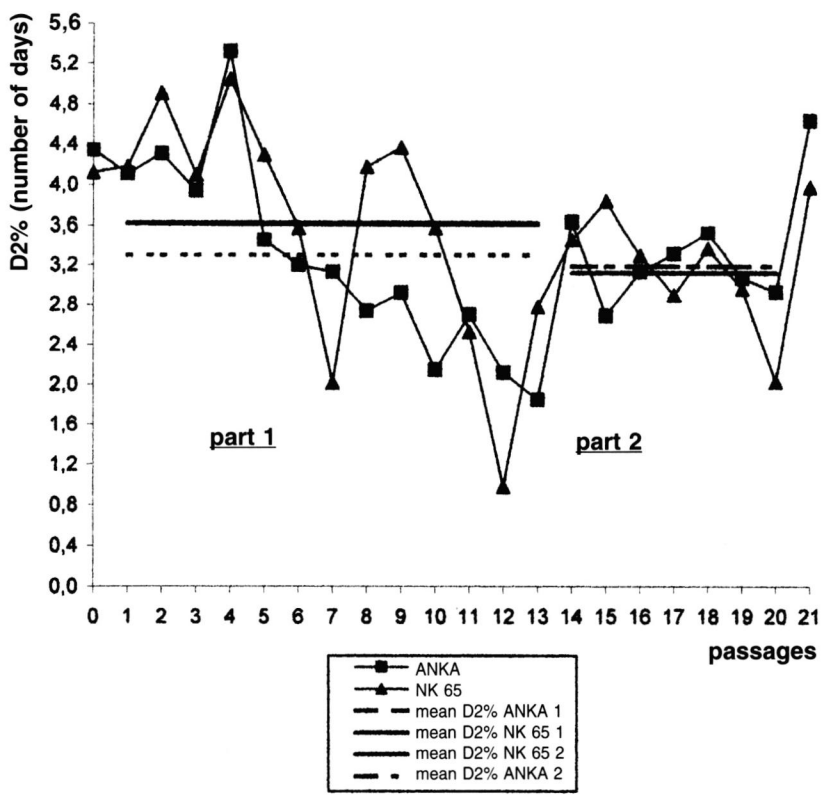

Fig. 1. – Evolution of 2 % delay time tests (D2%) according to the passages.

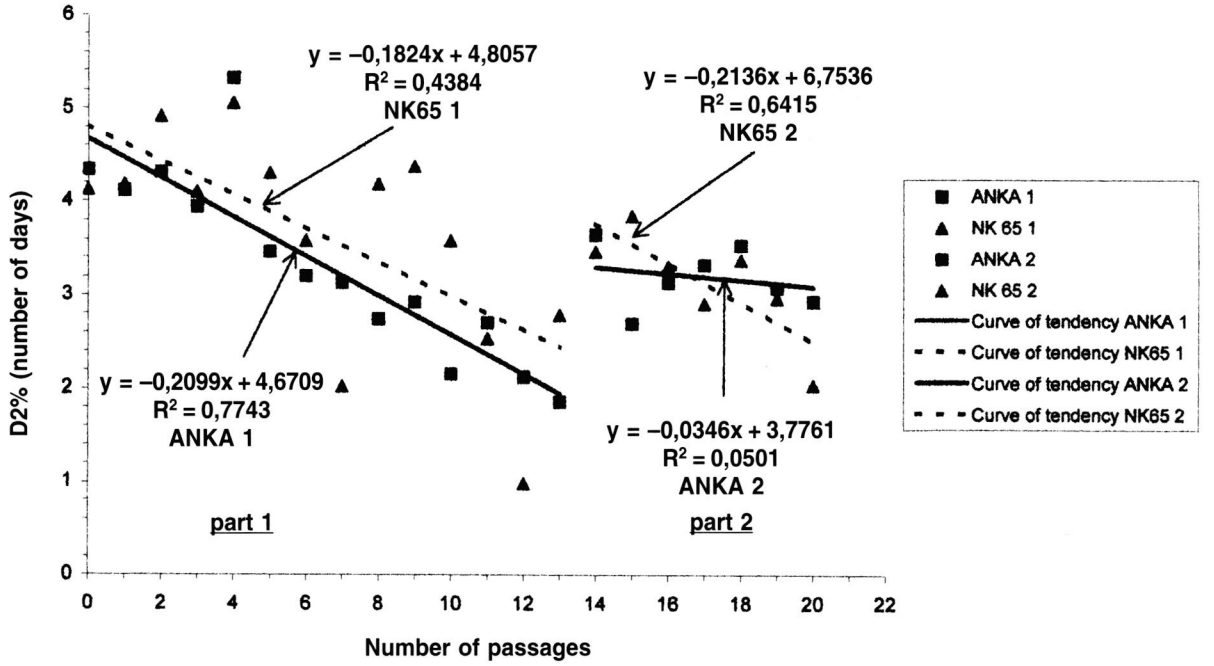

Fig. 2. – Curves of tendency of 2 % delay times tests (D2%).

At the tenth passage without chloroquine, D2% was 4.64 days (i.e. very closely related to the initial value: 4.34 days).

• *Plasmodium berghei* NK 65

The behaviour of this strain was appreciably different from that of ANKA strain. The change in D2% was very irregular; it was 4.12 days at passage 0 and 2.03 days at passage 20. Again, there were two phases. First, from passage 0 to passage 13, D2% varied from 4.12 to 2.78 days, with a means of 3.62 days. The gradient of this tendency curve was – 0.18, indicating a decrease in D2% comparable to that for ANKA. D2% then varied from 3.46 days in the 14th passage to 2.03 in the 20th passage, with a means of 3.12. The gradient of this tendency curve was – 0.21, what does not indicate a plateau phase, but a continuing decrease in D2%.
After ten passages without chloroquine, D2% returned to 3.98 days, i.e. very close to the initial value (4.12 days).

Change in the SI during the 20 passages and percentage of young forms

The percentage of each parasitic stage was calculated every six hours for two schizogonic cycles, to study the influence of drug pressure on the distribution of the parasite stages. The percentages of young forms (Rings and YTs) were much higher at the 20th passage (Fig. 3) than at the beginning of the experiment, especially in *P.b.* ANKA. The SI calculated for the initial strain was about 0.2 for ANKA, and about 0.2 for NK 65, values which were preserved until the 6th and the 12th (ANKA = 0.23, NK 65 = 0.23) passage. In the 20th passage, they reach 0.36 for ANKA, and 0.28 for NK 65 i.e. almost twice the initial values for ANKA. Both strains were then subjected to 10 successive passages without chloroquine pressure. The average SIs in the 10th passage is 0.16 for ANKA, and 0.18 for NK 65, indicating a return to the initial values.

DISCUSSION

Changes in the D2% of *P. berghei* ANKA and *P. berghei* NK 65

The ANKA strain became more resistant to chloroquine than the NK 65 strain (mean D2% of 3.27 for ANKA during the 20 passages, and mean D2% of 3.45 for NK 65). *P. berghei* ANKA is naturally more sensitive to chloroquine (5 mg/kg) than is *P. berghei* NK 65 (10 mg/kg, according to the scale established by Beauté-Lafitte *et al.*, 1994b). Therefore *P.b.* NK 65, naturally less sensitive than *P.b.* ANKA, reacts little to the chloroquine pressure. Thus the decrease of D2% for *P.b.* ANKA was greater under chloroquine pressure.
Peters (1970 and 1978) draw attention to the apparent reappearance of gametocytes in chloroquine-resistance lines selected from derivatives of *P. berghei* (K173), and then demonstrated that the process of drug pressure (the same technique we used in the present work) had

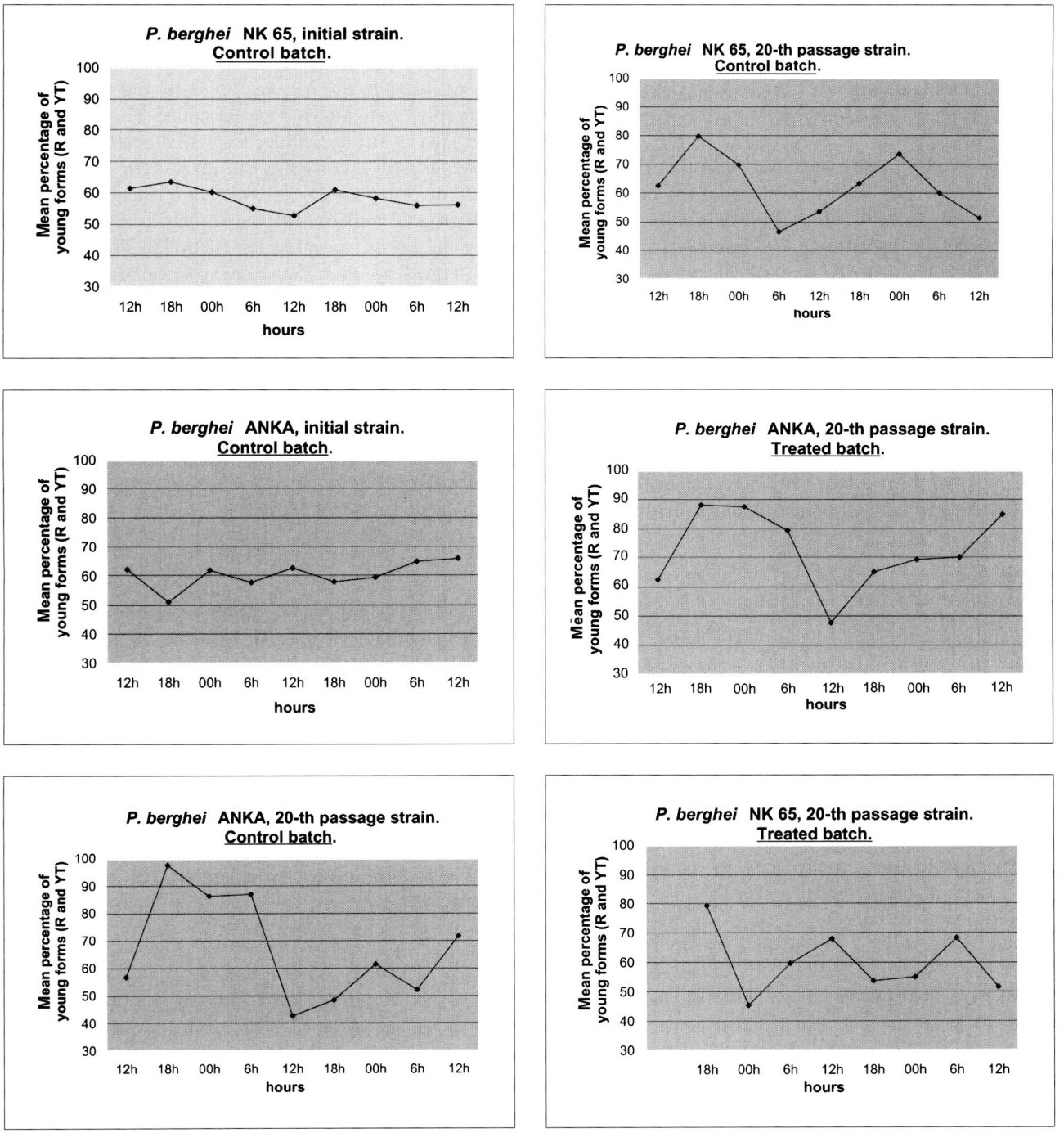

Fig. 3. – Mean percentage of young forms obtained during 48th with four mice.

selected a different parasite, now known as "*P. yoelii* ssp. NS". By this chloroquine pressure, Peters *et al.* (1978) selected lines with isoenzymes like those of *P. yoelii nigeriensis* from stock derived from the type strain of *P. berghei* (K173) and strain NK 65, both of which were originally isolated in Katanga. Hybridization of DNA (Chance *et al.*, 1978) and cross-immunity studies (Peters, 1978) indicated that *P. yoelii* ssp. differed not only from *P. berghei*, but also from *P. yoelii nigeriensis* and their conclusion was that the original isolate must have been a mixture which has survived for more than thirty years in the laboratory. No lines with enzymic similarities to *P. yoelii* were selected from *P. berghei* ANKA. Their conclusion was that there may be a relict population of *P. yoelii* ssp. which is sympatric with *P. berghei* in parts of Katanga... While Peters *et al.* (1978) gave reasons for considering that NS differed from *P. yoelii nigeriensis*, an unpublished study of George Snounou suggested that this parasite may be identical to *P. yoelii nigeriensis*. The progressive changes in the 2 % delay time observed in the present work bear a close similarity to those recorded by the Peters group but no changes in gametocytogenesis were noticed (probably because our NK 65 and ANKA already did produce gametocytes).

We do not believe that a similar occurrence arose from our experiments because in our work, the resistance was unstable in contrast with that of *P. y. nigeriensis* which is naturally the most drug-resistant of the rodent malaria parasites.

Changes in SI during the 20 passages, percentage of young parasite forms

The SI values were sharply higher at the 20th passage than in the initial strains (ANKA = 0.36, NK 65 = 0.28), indicating that the synchronicity increased. This was more marked for ANKA than for NK 65. The results therefore demonstrate:

- a decrease in D2%, i.e. an increase in *Plasmodium* resistance;
- an increase in the SI, i.e. an increase in synchronicity.

Landau *et al.* (1994) showed a positive correlation between the synchronicity of a strain and its sensitivity (notably for *P. yoelii nigeriensis)*. Our results also seem to differ from those obtained for *Plasmodium chabaudi chabaudi (*Coquelin *et al.*, 1997). These authors observed a correlation between the acquisition of chemoresistance under chloroquine pressure, and the decrease in the SI. These phenomena were explained by the theory of latent merozoites: the merozoite stage is totally resistant to chloroquine. Merozoites supply permanently parasitic stages that infect the blood. Chloroquine pressure results in the loss of cycles of non-latent merozoites (with immediate penetration), because the various stages of this cycle are sensitive to chloroquine. But every latent merozoite generates a cycle at a specific moment, and their accumulation causes a marked asynchronysm. The starting values return when chloroquine administration or subinoculations are stopped.

Can we explain the increase in SI by the existence of latent merozoites in our experiments? The two strains used have a strong affinity for reticulocytes (20 % for ANKA, and 50 % for NK 65, Deharo *et al.*, 1996), with a strong polyparasitism. This often causes the early explosion of infected RBC, resulting in:

- the failure of many cycles before the formation of old stages, so that there are predominantly young forms which influences the strain synchronicity;
- the massive destruction of infected RBC causes considerable neoreticulocytosis;
- the new reticulocytes are invaded by the available merozoites at this precise moment (in NK 65 strain there are more latent merozoites (synonyms of asynchronism) than in ANKA strain (Beauté-Lafitte *et al.*, 1994b; Deharo *et al.*, 1996)). Hence, NK 65 should be more asynchronous than ANKA under the effect of chloroquine. We indeed find that NK 65 is less synchronous (0.28) than ANKA (0.36) at the 20-th passage. The synchronicity of both strains was increased after 20 passages. It is linked to the increase in young forms. Chloroquine also effects the latent merozoites selection, but this is masked by parasites preference for reticulocytes and its consequences for the biological cycle. Our results do not differ markedly from those of Deharo, or those of Beauté-Lafitte. The difference between our findings and those of Coquelin can be explained by the difference between *P. chabaudi* and *P. berghei* within host cells. Peters (1965) with *P. berghei* and Coquelin *et al.* (1997) with *P. chabaudi* also showed that the physiological parameters modified by chloroquine pressure (SI, D2%) return to their initial values when chloroquine is removed; our own experiments confirm this.

The average values of SI were 0.16 for ANKA and 0.19 for NK 65 after 10 passages without chloroquine pressure, so there was a return to the starting values (0.2 for ANKA and NK 65). This is probably due to the disappearance of the drug pressure, which stops the selection of merozoites, and decreases the percentage of young forms. The percentages of each parasite stage were the same as the initial percentages of both strains. These results are confirmed by D2% values, that are equivalent between the initial strains and the 10th passage without chloroquine values (4.64 days for ANKA and 3.98 for NK 65).

Otherwise, the infection develops little, and the blood parasite concentrations remain low, because the parasites too tightly enclose in the RBC cannot mature. *P.b.* ANKA is also a mortal strain for mice, even at low (about

20 %) parasite concentration, because multiinfected reticulocytes (up to seven parasites per cell), become hypertrophic, accumulate in the brain capillaries, hinder the blood circulation and finally kill the animal.
Peters (1968) showed, in the NK 65 strain, an association between the acquisition of chloroquine resistance and the decrease in the parasite virulence in white mice. We also find a return to normal sensitivity when the chloroquine pressure stops: this return is accompanied by an increase in virulence.

CONCLUSION

The *P. berghei* lines NK 65 and ANKA react in an appreciably differently way (synchronicity, chemoresistance acquisition, and percentage of every parasitic stage) to drug pressure. Both strains were of the same *Plasmodium* species, and the experimental protocol was designed to limit individual variations of reactions of the host: mice of the same litter, coming from the same delivery, were treated simultaneously. It is obviously quite different in nature. This study may give a general survey of the difficulties encountered in field studies on human patients, infected by several clones (or several species!) at different times.

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