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ORIGINAL PAPER

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The erythrocytic schizogony of two synchronized strains of *Plasmodium berghei*, NK65 and ANKA, in normocytes and reticulocytes

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Abstract By a modified Percoll-glucose centrifugation technique the rings and young trophozoites of two strains of *Plasmodium berghei,* NK65 and ANKA, were separated from the other erythrocytic stages and inoculated into mice. The subsequent infection was followed for ANKA in normal mice and for NK65 in normal mice and in mice with high-grade reticulocytosis induced by injections of phenylhydrazine. The duration of the erythrocytic schizogony of the NK65 strain was shown to be independent of the age of the host cell, and the hour of inoculation did not influence the cycle of the ANKA strain.

Introduction

Since its first description by Vincke and Lips in 1948, hundreds of publications on *Plasmodium berghei* have appeared in the literature. Many strains have been isolated and biological differences between strains have been observed, such as differences in virulence, sensitivity to drugs, and preference for invasion of normocytes or reticulocytes.

One of the main peculiarities of the parasite is its preference for immature red blood cells (Vincke and Lips 1948; Gaillard 1949) and its tendency to polyparasitize reticulocytes. Together with all the other species of murine malaria parasites, it was considered by Landau et al. (1979) as belonging to the "vivax group", and many efforts were made to adapt it to in vitro cultures. This objective was never totally achieved, and the parasite's preference for reticulocytes was considered to be responsible for the difficulties met in trying to obtain a continuous culture (Mons et al. 1983; Ramaiya et al. 1987; Suhrbier et al. 1987).

According to Büngener (1985), the duration of the erythrocytic cycle depends on the age of the host cell and is shorter in mature than in immature red blood cells (RBCs). To verify this hypothesis and evaluate the possible impact on the parasitic rhythm, we synchronized by a modified Percoll-glucose technique (Deharo et al. 1994) the parasitemia of two strains of *P. berghei,* NK65 and ANKA. The former has a much higher affinity for immature RBCs than does the latter. For further differentiation of the strains, high-grade reticulocytosis was induced in mice infected with strain NK65 by injections of phenylhydrazine. Another parameter investigated was the influence of the time of inoculation on the duration of the erythrocytic schizogony of strain ANKA.

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Materials and methods

Strains

Plasmodium berghei NK65 (NK for New York-Katanga) was isolated from *Anopheles dureni millecampsi* in Katanga (Republic of Zaire) in the forest gallery on the river Kisanga near Lumbumbashi, and *P. berghei* ANKA (ANKA for Anvers-Kasapa) was isolated from the same host in Kasapa, not far from the first locality (Wery, personal communication).

Infection of mice

Random-bred Swiss albino mice weighing 20–25 g (Iffa-Credo, France) were inoculated intraperitoneally with blood from a frozen aliquoted stock. When parasitemia reached at least 10%, mice were anesthetized and the blood was collected by retro-orbital puncture into heparin-containing test tubes. The blood was centrifuged for 5 min at 1,200 *g* and the supernatant was discarded. The cell pellet was resuspended in 3 vol. phosphate-buffered saline (PBS) containing 0.3 *M* D-glucose (PBS-G).

Preparation of the Percoll suspension

The separation technique previously described by Deharo et al. (1994) was modified as follows:

70% Percoll was obtained by mixing 35 ml Percoll (Pharmacia, Paris, France), 5 ml of 10-fold concentrated PBS-G, and 10 ml distilled water. Next 15-ml Nalgene tubes were filled up to 6 ml with the 70% Percoll suspension and overlaid with 1.5 ml cell suspension. The tubes were centrifuged for 7 min at 30,000 *g* in a fixed-angle rotor prewarmed to 28°C. The parasites were fractionated by the gradient into two distinct cell bands: fraction 1 (the upper layer), comprising mainly mid-term trophozoites, old trophozoites and schizonts (parasitemia, approx. 95%); and fraction 2 (the lower layer), comprising mainly rings, young trophozoites, and uninfected erythrocytes (parasitemia, 1% or less). The latter fraction was removed by direct puncture through the wall of the tube and diluted in 5 vol. PBS. The suspension was centrifuged for 5 min at 1,200 *g* and the cell pellet was resuspended in saline for the injection of 0.5 ml into mice by the intravenous route (i.e., 10^6 parasites).

Follow-up of parasite development

Blood smears from the infected mice were prepared at 3-h intervals for 45 or 51 h consecutively, fixed with methanol, and stained with Giemsa. The total parasitemia (percentage of infected cells) and the parasitic pattern (percentage of each stage) were evaluated.

Parasitic pattern

Five stages were defined as follows: the ring (R), the smallest intracellular stage observed following invasion of the erythrocyte by the merozoite; the young trophozoite (YT), displaying a larger vacuole, a more developed cytoplasm, an irregular contour, and very faint (if any) pigment; the midterm trophozoite (MT), occupying one-third to one-half of the host erythrocyte volume and displaying a larger nucleus, relatively more abundant cytoplasm, a smaller vacuole, and a few granules of pigment; the old trophozoite (OT), occupying from one-half to almost the entire volume of the RBC and showing densely staining cytoplasm, a small vacuole, and profuse pigment; and schizonts (S), which were scanty and were thus counted with OTs.

Phenylhydrazine treatment

For generation of strong reticulocytosis (30%), 70 mg/kg of a phenylhydrazine solution in water was inoculated into mice by the intraperitoneal route. The compound was injected into mice 2 days before the inoculation of parasites.

Results

Course of infection of *Plasmodium berghei* ANKA

The follow-up of the parasitemia and parasitic pattern of *P. berghei* ANKA synchronized and inoculated either at midday or at midnight is depicted in Fig. 1.

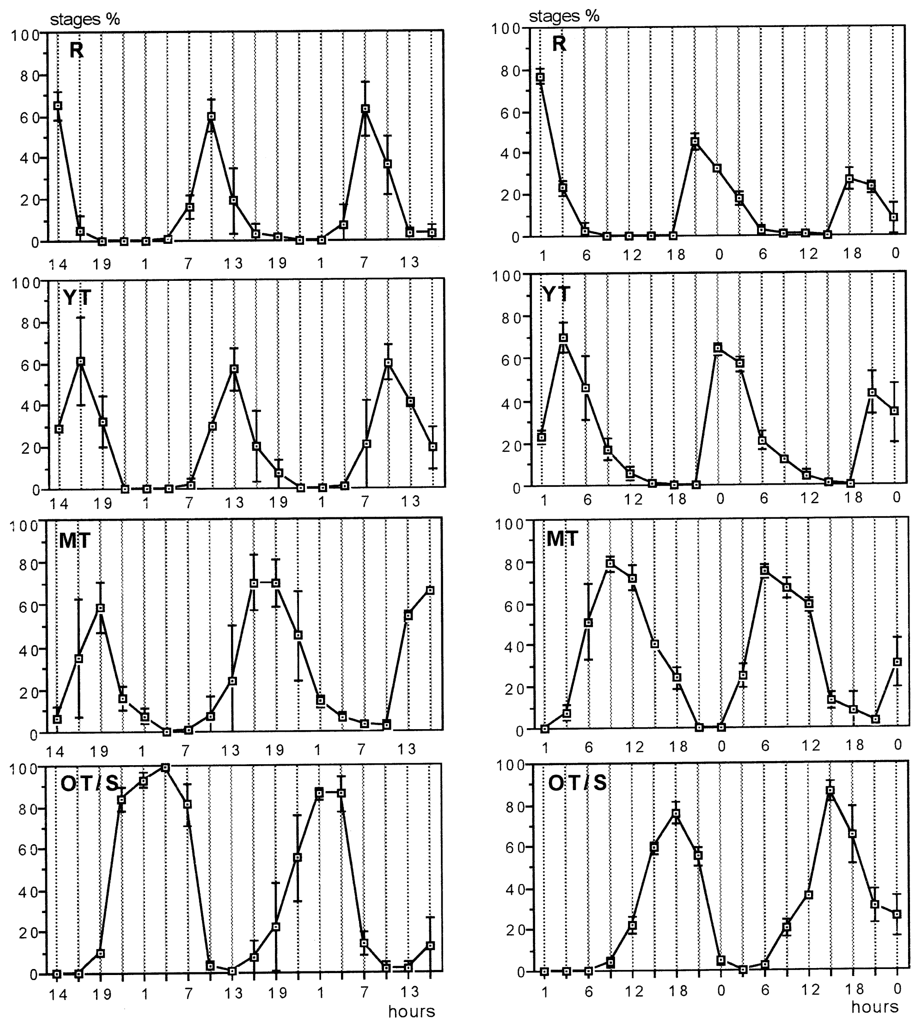

Fig. 1 Follow-up of the parasitic pattern (percentage of each stage) of *Plasmodium berghei* ANKA inoculated at 1300 hours (*left*) or at midnight (*right*) as evaluated at 3-h intervals. Each value represents the mean percentage for 3 mice (*R* Ring, *YT* young trophozoite, *MT* midterm trophozoite, *S* schizont, *OT* old trophozoite)

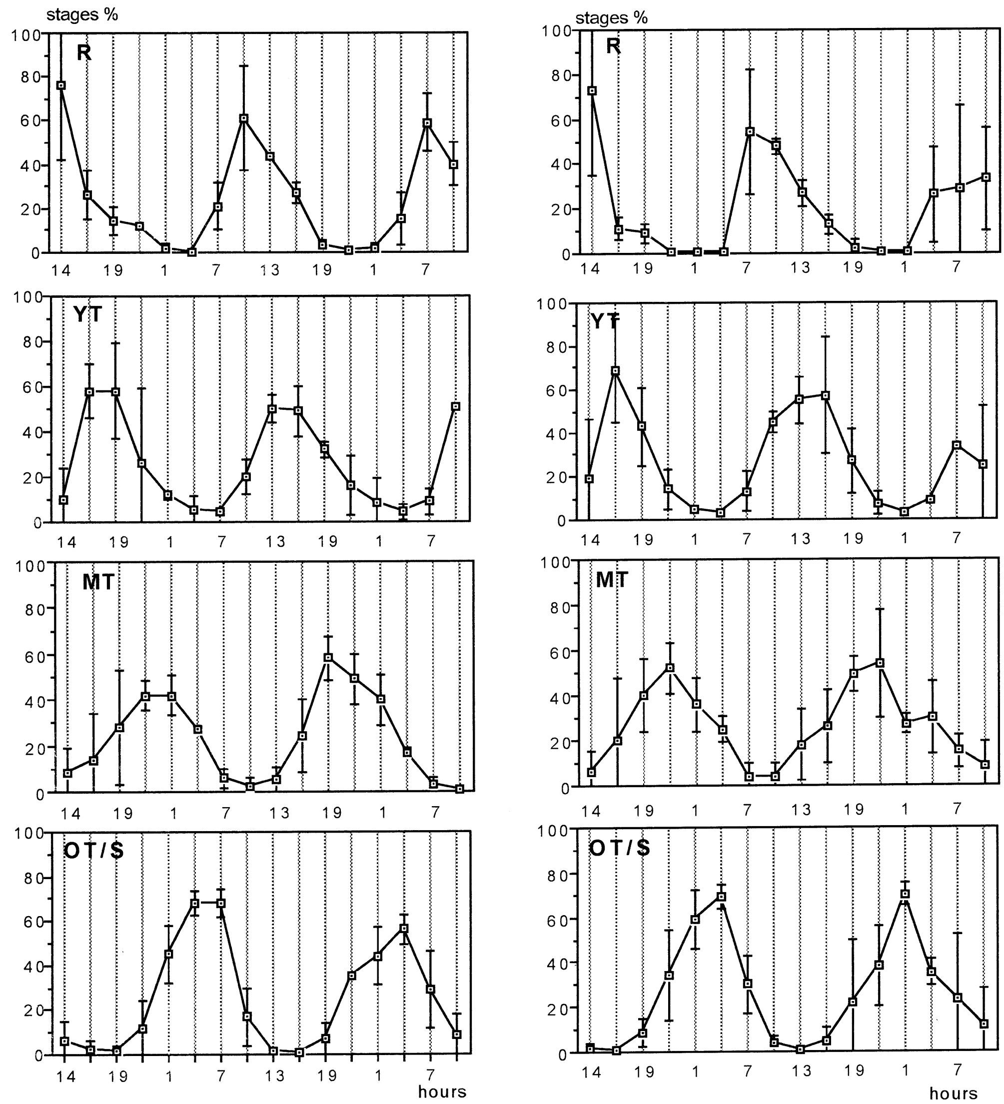

Fig. 2 Follow-up of the parasitic pattern (percentage of each stage) of *P. berghei* NK65 with phenylhydrazine (*left*) and without (*right*) as evaluated at 3-h intervals. Each value represents the mean percentage for 3 mice

Inoculation at 1300 hours

At 1400 hours, 1 h after the injection of the Percoll layer with the young synchronized parasites, 65% of the parasites were at the R stage and 29% were at the YT stage. From then on, the percentage of Rs decreased rapidly while increasing numbers of YTs appeared, reaching a peak at 1600 hours. The following stages peaked successively: MTs, at 1900 hours and OTs and Ss, at 0400 hours. The second peak of each stage was observed at 21–23 h after the first peak, i.e., Rs peaked at 1000 hours; YTs, at 1300 hours; MTs, at between 1600 and 1900 hours; and OTs and Ss, at 0100 hours. After that the infection became desynchronized.

Inoculation at midnight

The parasitic pattern observed in mice inoculated at midnight was very similar to that seen in mice inoculated at midday, the interval between two peaks of the same stages being 21–23 h. In the two groups of mice, rings remained detectable at 6 h after the inoculation.

Course of infection of *P. berghei* NK65

The follow-up of parasitic patterns of *P. berghei* NK65 in control mice and mice pretreated with phenylhydrazine is depicted in Fig. 2. The parasitic pattern of strain NK65 in normal untreated mice inoculated at 1300 hours was very similar to that of ANKA, the interval between peaks of stages being 21–23 h. Rings remained detectable at 12 h after the beginning of the experiment in the treated mice and at 9 h in the untreated mice.

Affinity of *P. berghei* for reticulocytes

In the normal untreated mice, strain ANKA spontaneously showed a less marked attraction to reticulocytes (20%)

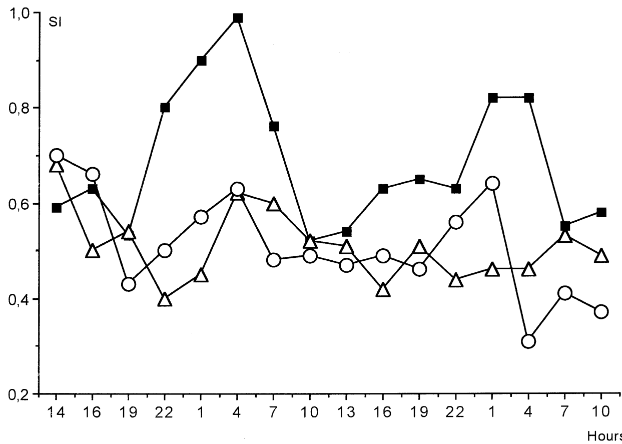

Fig. 3 Evolution of the synchronicity index (*SI*) as evaluated at 3-h intervals. Each value represents the mean percentage for 3 mice (*Black squares* ANKA, *white triangles* NK65 with phenylhydrazine, *white circles* NK65 without phenylhydrazine)

than did strain NK65 (50%; data not shown). When the latter infected mice had been treated with phenylhydrazine, 85% of parasites developed inside reticulocytes. At the beginning of the experiment the levels of parasitemia were similar (0.1%) in control and phenylhydrazine-treated mice. At the end of the follow-up period, 42 h later, the multiplication rate was X5.5 in control mice and X10 in treated mice (data not shown).

Synchronicity index

A synchronicity index (SI; Fig. 3) was calculated as follows:

SI = Standard deviation (SD) of the percentage of each stage divided by 50 (50 being the SD in a 100% synchronous infection, counting 4 different stages). This index varies from 0 (asynchronicity) to 1 (synchronicity). NK 65 showed a very similar SI in normal or pretreated mice (SI, between 0.3 and 0.7). ANKA was much more synchronous than NK65 when the OTs and Ss peaked (SI, 0.8–1).

Discussion

Synchronization techniques

The technique used in this work was basically the same as that previously described by Deharo et al. (1994) for synchronization of *Plasmodium yoelii*. It is based on the variations in erythrocytic density determined in a hypertonic medium by the maturation and the subsequent increase in the permeability of the membranes.

The centrifugation with a Percoll-glucose suspension enables the separation of the very young parasites (Rs and YTs gathered in fraction 2) from the other stages and the inoculation of a single parasitic stage. However, when several Rs and YTs are virtually inside polyparasitized reticulocytes, they migrate into the layer containing old stages. In the case of strain NK65, the multiple invasion of reticulocytes occurs very early in the infection and very few young stages are collected in fraction 2 of the gradient. In this respect, NK65 differs from all *P. yoelii* strains and from ANKA. To obtain a sufficient number of Rs and YTs of NK65, it is necessary to perform the separation at a relatively early time during the infection and to use larger numbers of mice. The use of a single Percoll concentration instead of a gradient as originally described by Deharo et al. (1994) speeds up the duration of the procedure to a period of only a few minutes.

Duration of the schizogonic cycle

The interval of 21 h between two peaks of Rs or YTs did not vary when mice were inoculated with strain ANKA at 1200 hours or midnight. These results confirm those of Mons et al. (1985), who combined a culture of the same strain up to the mature schizont stage with their inoculation into young rats previously treated with phenylhydrazine. The infection was thus synchronized and the authors found a duration of 22–23 h for the schizogonic cycle.

According to Büngener (1985), the age of the parasitized cell determines the duration of the erythrocytic cycle. The development of *P. yoelii* would last 17 h inside normocytes and 25–29 h inside reticulocytes. The present study showed that the length of the cycle of *P. berghei* did not vary when high-grade reticulocytosis was induced by phenylhydrazine treatment. It appears that the duration of the schizogonic cycle of the two strains of *P. berghei* with a different affinity for reticulocytes is similar and independent of the rate of invasion of reticulocytes.

Multiplication rate

It has been shown by various authors that the rate of multiplication of strains of *P. berghei* is dependent on the level of reticulocytosis. An increase in the numbers of reticulocytes inducted by hemolysis following the injection of phenylhydrazine (Ott 1968) or of anti-RBC serum (Schwink 1960) enhances the infection, whereas a drop in the production of RBCs caused by medullar irradiation (Singer 1953), hypertransfusion (Ladda and Lalli 1966), or administration of corticoids (Cox 1974) depresses the parasitic development.

Our results are compatible with the following interpretation: the multiplication rate of NK65 in mice with normal reticulocytosis is 2 times lower than that in mice with increased reticulocytosis. This is not necessarily the case, i.e. *P. yoelii nigeriensis*, which is the most virulent subspecies of the *P. yoelii* group, is also the one with the lowest affinity for reticulocytes.

Asynchronicity

Previous work on the duration of the schizogonic cycle of *P. yoelii* (Deharo et al. 1994) showed that after the inoculation of the gradient layer containing young parasites, new rings were detectable during the following 12 h, indicating that merozoites were present in the inoculum and that they penetrated progressively into the erythrocytes. For strain NK65, more rings were seen during the first 12 h post-inoculation than were observed for ANKA, and this may account for the better synchronization of the latter.

These results confirm observations by Beaute-Laffite et al. (1994), who showed the numbers of latent merozoites to be greater for strain NK65 than for strain ANKA. They also thought that the biology of merozoites and its impact on the degree of synchronicity was responsible for the differences in sensitivity to chloroquine found between the two strains.

The duration of the erythrocytic cycle, i.e., 18 h for *P. yoelii*, 21 h for *P. berghei*, and 24 h for *P. chabaudi* and *P. vinckei*, is an important parameter that should be taken into account in performing chronotherapy studies and comparing the activity of drugs in different malaria models.

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