DNA synthesis in malaria parasites during sexual and erythrocytic asexual development : proefschrift ... / door Christoffel Jan Janse.

DNA SYNTHESIS IN MALARIA PARASITES DURING SEXUAL AND ERYTHROCYTIC ASEXUAL DEVELOPMENT

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Contents

Chapter I

GENERAL INTRODUCTION

1. Outline of this study

The micro-organisms causing malaria, one of the most important parasitic diseases in the world, are commonly referred to as malaria parasites. These protozoa are found in mammals, particularly primates and rodents, and in birds and reptiles. They are all grouped in one single genus Plasmodium. According to the most recent classification of the animal subkingdom Protozoa, edited by the Society of Protozoologists (Lee et al., 1985) malaria parasites belong to the phylum Apicomplexa (formerly Sporozoa), of which all members are parasitic, and the order Eucoccida. Common characteristics of this order are a life cycle consisting of an alternation of asexual multiplication by schizogony and sexual reproduction by anisogamy.

The life cycle of all mammalian malaria parasites is basically the same. It comprises a sexual phase - starting with the formation of micro- and macrogametocytes in the erythrocytes of the vertebrate host and is completed by formation of gametes and subsequent fertilization when these gametocytes are ingested by a mosquito of the genus Anopheles - and several types of asexual multiplication by schizogony both in the vertebrate host and in the mosquito (see chapter I.3 for details). The complex life cycles of mammalian malaria parasites have been described in detail at the light and electronmicroscope level (reviewed by Garnham 1966, 1980; Aikawa and Seed, 1980; Sinden, 1983).

In spite of many studies dealing with the biochemistry of parasites (reviewed by Sherman, 1979; Homewood & Neame, 1980) and increasing knowledge about the molecular structure of genes coding for antigens (reviewed by Kemp et al., 1986), the genetic and molecular processes underlying and directing the extensive cyclic differentiation are still poorly understood. This lack of knowledge can mainly be attributed to technical difficulties in cultivation and purification of the different developmental stages. During the last few years, however, considerable progress has been made with the in vitro cultivation of erythrocytic and exo-erythrocytic stages and with

the development of techniques for in vitro sexual development (reviewed by Trigg, 1985).

For studies on the genetics and on the molecular basis of cell differentiation it is most important to understand how the genetic material is organized during the life cycle, e.g. the chromosome number, the ploidy of each parasite stage, the time and rate of DNA synthesis, the time at which meiosis occurs, etc. Several cytological studies have been made on the genome of malaria parasites but disappointingly little information on these points has been obtained (see chapter I.2). This is mainly due to the unusual condensation pattern of the parasitic DNA. Chromosomes of malaria parasites do not condense prior to mitosis (Sinden, 1978), which precludes easy establishment of molecular karyograms by conventional cytological techniques.

In the present study we determined, as a first step towards a better understanding of the organization of the genetic material of malaria parasites, the ploidy of the successive developmental stages and the time and rate of DNA synthesis during asexual and sexual development, using cytophotometric methods.

In contrast to biochemical methods, cytophotometry allows the measurement of the DNA content of individual cells, that can be identified morphologically, thus avoiding sources of errors related to purification of cells, cell synchronization, inaccuracy of cell counting and loss of DNA during extraction. We mainly used the Feulgen-pararosaniline reaction procedure. Although the physicochemical background of the Feulgen staining reaction has not been completely elucidated, it is highly specific and - when properly controlled - stoichiometric for DNA. Therefore it has become a valuable tool and one of the most widespread methods in quantitative cytophotometry (see Schulte (1986) for staining properties). Moreover, it has been shown to be sufficiently sensitive to be applicable to organisms with very small genome sizes (Cornelissen et al., 1984).

This study is confined to DNA synthesis during erythrocytic asexual and erythrocytic as well as post-erythrocytic sexual development.

The intra-erythrocytic haploid parasites reproduce asexually by repeated mitotic nuclear divisions (schizogony) resulting in multinuclear schizonts which, by multiple cell fission, produce the next generation of haploid parasites. Time and rate of DNA synthesis during this process has been investigated biochemically, but the results of different studies are contradictory (Homewood and Neame, 1980; chapter I.2).

Intra-erythrocytic parasites can also start to differentiate into sexually committed 'male' and 'female' cells, called micro- and macrogametocytes, respectively, which can transform into gametes within the midgut of a vector mosquito. The factors governing this intra-erythrocytic differentiation (gametocytogenesis) are largely unknown (Mons, 1985). Sex itself is not determined by sex-specific chromosomes since cloned haploid parasites can produce both micro- and macrogametocytes (Down, 1947; Inselburg, 1983).

The DNA content of sexual cells has never been measured directly, but results of electron microscope studies and experiments in which mitomycin-C was used as a specific DNA synthesis inhibitor strongly suggested that both male and female gametocytes replicate their genome during early development in the red blood cell (Toyé et al., 1977; Sinden and Smalley, 1979; Sinden, 1982). Macrogametocytes were thought to pass through a transiently diploid stage, followed by so-called cryptomitosis in order to produce haploid mature cells, while mature microgametocytes were considered octoploid, giving rise to 8 haploid microgametes by repeated mitosis during exflagellation in the mosquito midgut. Very recently Sinden and Hartley (1985) demonstrated that meiosis takes place within several hours after fertilization. If meiosis in malaria parasites follows a normal eukaryotic pattern one may expect that the young zygote replicates from a diploid to a tetraploid value.

At the start of this study, the main problem was to obtain the different sexual stages in sufficient number. The major part of the sexual development occurs in the less accessible mosquito midgut and has been incompletely described. Moreover, little was known about developmental and

survival time, time of maturity and duration and degree of infectivity of the intra-erythrocytic gametocytes (see review by Sinden, 1983). Accurate estimation of the time and rate of DNA synthesis during the different phases of sexual reproduction is not feasible as long as one is dependent on mere morphology of stages and on non-synchronized infections in which many of the stages occur simultaneously. Therefore, we first developed methods for in vitro cultivation and synchronization of the rodent malaria parasite P. berghei (section 1: chapter I - VI). This enabled us to study sexual development in detail and to obtain rather pure preparations of successive stages for DNA measurements, as described in Section 2 (chapter VII - XI).

In chapter II and III methods are described for the in vitro cultivation of the erythrocytic stages of P. berghei. These methods enabled us to study schizogony, which normally is confined to blood capillaries of the host, and gametocytogenesis under standardized conditions.
In vivo and in vitro synchronization of erythrocytic schizogony and gametocytogenesis is described in chapter IV.
Using parasites from these synchronized infections and in vitro cultures we have been able to determine precisely the DNA content of mature gametocytes and the time and rate of DNA synthesis during asexual development (chapter VIII and XI).
Young gametocytes of P. berghei cannot be distinguished from asexual trophozoites (chapter IV), which hampers study of DNA synthesis during early gametocytogenesis. This process, therefore, was studied in the human parasite P. falciparum, where gametocytogenesis takes a much longer time and young gametocytes are readily distinguished from asexual parasites (chapter IX).
Chapter V describes a standardized method for in vitro fertilization and zygote development of P. berghei. The in vitro development of macrogametes into ookinetes is compared to that in vivo in chapter VI. The in vitro method has been used to test functional maturity of in vivo and in vitro produced gametocytes and to measure DNA contents of parasites

during gamete formation, fertilization and zygote development (chapter VII, VIII, X).

Besides microscopic microfluorometry of individual cells on slides, cell by cell measurements of DNA contents can also be performed by flow cytometry, a simple and rapid method if cells can be obtained in sufficient number. Results of DNA measurements in malaria parasites, obtained with this method, are shown in chapter XI.

2. The DNA of malaria parasites

Genome size

The total amount of DNA of the haploid genome, expressed in picograms (pg) or in base pairs (bp), is a characteristic of each living species, known as genome size, genome complexity or C-value.

Table 1 shows estimates of genome sizes of different Plasmodium species. The estimates vary widely, even for a single species, and range from 1 x 10^7 to 3.8 x 10^8 bp.

The genome size of 2.5 x 10^7 bp, established for P. berghei by comparison of sporozoites with human chromosome 21 using highly standardized methods, is probably the most accurate estimation (Cornelissen et al., 1984). It is about 6 times the genome size of Escherichia coli, twice that of yeast, and less than one hundredth of the human haploid genome.

Base composition of DNA

Base composition of the nuclear DNA differs between the various mammalian parasites. The guanine - cytosine (G+C) content of P. falciparum (Goman et al., 1982; Pollack et al., 1982; McCutchan et al., 1984; Williamson et al., 1985), P. berghei, P. vinckei and P. chabaudi (McCutchan et al., 1984) has been reported to be around 18% which is considerably lower than the G+C content of host DNA (37%). The slightly higher values (23-24%) for P. berghei and P. vinckei found in earlier investigations (Gutteridge

Table 1: Estimates of the genome size of different Plasmodium species.

Plasmodium species	genome size in in base pairs	reference
P. falciparum	1-2 x 10^7 bp	Goman et al. (1982)
	2 x 10^7 bp	Pollack et al. (1982)
	3.8 x 10^8 bp	Hough-Evans and Howard (1982)
P. knowlesi	1.9 x 10^7 bp	Gutteridge et al. (1971)
P. yoelii	1.5 x 10^7 bp	Dore et al. (1980)
		Birago et al. (1982)
		Casaglia et al. (1985b)
P. berghei	1.5 x 10^7 bp	see ref. P. yoelii
	2.5 x 10^7 bp	Cornelissen et al. (1984)
	4.8 x 10^7 bp	Bahr (1966)

et al., 1971; Dore et al., 1980) probably can be attributed to contamination with host DNA (McCutchan et al., 1984). In comparison to the former species the primate parasites P. knowlesi and P. fragile showed a much higher G+C content: 30-38% (Gutteridge et al., 1971; McCutchan et al., 1984; Williamson et al., 1985) and 30% (McCutchan et al., 1984), respectively, while the DNAs of P. cynomolgi and P. vivax consist of two components, one with a low (about 20%) and the other with a high (about 30%) G+C content (McCutchan et al., 1984).

Repetitive DNA

About 10% of the DNA of P. falciparum consists of repetitive sequences (Hough-Evans and Howard, 1982). In P. berghei and P. yoelii repDNA fractions were found of 5-11% and 9-17%, respectively (Dore et al., 1980; Birago et al., 1982; Casaglia et al. 1985b). In cloned lines of the latter species this fraction decreased in parallel with loss of infectivity of gametocytes, suggesting a causal relationship between both. On the other hand, in P. falciparum no relationship was observed between prevalence of repDNA sequences and the parasite's potential for sexual differentiation (Bhasin et al., 1985).

Restriction enzyme analyses of total DNA of P. falciparum showed significant differences in the arrangement of repetitive sequences between isolates from different geographical origins and between clones from a single isolate (Goman et al., 1982; Bhasin et al., 1985; Oquendo et al., 1986). Even within a cloned line the arrangement of repDNA sequences changed after a six month culture period (Bhasin et al., 1985). Cloned repetitive sequences from P. falciparum did not cross-hybridize with DNA of other malaria species (Guntaka et al., 1985; Aslund et al., 1985; Bhasin et al., 1985; Cornelissen et al., 1985; Oquendo et al., 1986).

Extra-nuclear DNA

Mitochondrial DNA has been isolated from P. yoelii and consisted of closed circular DNA molecules with a length of 10.5 μm (Dore et al., 1983). Similar DNA molecules were found in P. knowlesi, roughly representing about 1% of the organisms total DNA (Williamson et al., 1985). Circular DNA has not yet been detected in P. falciparum (Goman et al., 1982).

Chromatin structure

Little is known about the structural organization of the DNA of malaria parasites. Conflicting results have been reported so far on the occurrence of histones in malaria parasites. In sexual cells of Plasmodium no histones could be detected by cytochemical methods (Sinden et al., 1983) and purified Plasmodium DNA digested with micrococcal nuclease did not show a

nucleosome banding pattern nor did Plasmodium DNA hybridize with DNA probes from rat and from sea urchin coding for histone H_4 (Frontali, 1985). Wunderlich et al. (1980), however, showed that, at the electronmicroscope level, chromatin of intra-erythrocytic stages of P. knowlesi has a nucleosomal organization similar to that of higher eukaryotes.

Electron microscope studies have failed to detect condensed chromosomes during mitosis (Aikawa & Seed, 1980). As a result our knowledge of the chromosomal organization of the plasmodial genome is but poor. The number of kinetochores, found during mitosis of haploid stages of rodent malaria parasites, ranging from 8 to 10, was considered to be indicative of the number of chromosomes (Sinden, 1983). Very recently condensed chromatids were observed in electron microscope pictures of meiotic division of P. berghei zygotes; their maximal number was also estimated to be about 8 to 10 per cell (Sinden and Hartley, 1985).

A recently developed technique, pulsed-field gradient (PFG) gel electrophoresis, allows the separation of DNA molecules of 30 - 30.000 kilo bases (kb), a range including the sizes of most or all intact chromosomes of eukaryotes such as yeast (Schwarz and Cantor, 1984) and trypanosomatids (Van der Ploeg et al., 1984). It has been shown that breakage of chromosomes can be avoided, indicating that the separated molecules represent the DNA of full length chromosomes. Using this method Kemp et al. (1985) separated at least seven discrete chromosomal DNA molecules from P. falciparum. These DNA molecules varied in size by up to 20% between different isolates and were in the range of 0.8 - 3 Mbp. Two of the chromosomes seemed to contain the same sequences. Similar results have been obtained by Van der Ploeg et al. (1985) which separated at least 7 chromosomes of 0.7 to 2 Mbp. Some of the DNA molecules remained in the slot of the gel, suggesting that there are in addition one or a few chromosomes of larger size.

In P. falciparum and P. berghei no differences in PFG-karyotypes have been found between asexual and sexual stages (gametocytes, gametes) obtained from the same line or clone. Karyotypes of clones which differ in

gametocyte production varied significantly (Van der Ploeg et al., 1985; C. Frontali and M. Ponzi, pers. comm.).

DNA synthesis

Malaria parasites do not use exogenous pyrimidines, in contrast to purines, for nucleic acid synthesis (for review see Homewood and Neame, 1980), but are dependent on de novo synthesis of pyrimidines.

Almost all studies on DNA synthesis in malaria parasites deal with time and rate of incorporation of radio-labeled precursors into DNA during asexual intra-erythrocytic development. Gutteridge and Trigg (1970) and Jung et al. (1975) did not find a clear periodicity, but most other workers using synchronized infections demonstrated that DNA synthesis starts during the later part of trophozoite development and is highest during schizogony (Conklin et al., 1973; Newbold et al., 1982; Sodeinde & Luzzatto, 1982; Inselburg et al., 1984; Gritzmacher et al., 1984; Waki et al., 1985). DNA synthesis in this phase could be inhibited by aphidicolin (Inselburg et al., 1984), a specific inhibitor of polymerase-α (Ikegami et al., 1978), the enzyme primarily responsible for DNA chain elongation in all kinds of eukaryotes (Scovassi et al., 1980). This suggests that Plasmodium has a similar DNA polymerase.

DNA synthesis during sexual reproduction has never been demonstrated directly. It has been suggested that both sexes of gametocytes replicate their genome during early development, resulting in transiently diploid female and in octoploid male gametocytes, since young gametocytes of P. falciparum contain intra-nuclear microtubules (Sinden, 1982) and are killed by the DNA synthesis inhibitor mitomycin-C (Sinden & Smalley, 1979), while exflagellation in P. yoelii is insensitive to it (Toyé et al., 1977).

The gametes of both sexes are assumed to be haploid as a result of so-called cryptomitosis in the intra-erythrocytic female gametocyte (Sinden, 1983) and of rapid segregation of the 8 preformed haploid genome complements in the male gametocyte during exflagellation.

Mitosis

Mitosis occurs in different phases of the life cycle of malaria parasites: in the exo-erythrocytic and erythrocytic schizonts, during microgamete formation and in the oocysts. Mitosis in these protozoans is different from that found in higher eukaryotes: chromosomes do not condense, the nuclear membrane remains intact during mitosis and spindle fibers are formed inside this membrane (Aikawa and Seed, 1980). Moreover, activated microgametocytes, exo-erythrocytic schizonts and oocysts have large polyploid nuclei, containing numerous spindles. Nuclear fission in these stages waits until just prior to the so-called budding-off of microgametes, merozoites and sporozoites, respectively. During erythrocytic schizogony a normal mitotic sequence of genome segregation and nuclear fission is assumed to occur.

Meiosis

Malaria parasites have for long been considered haploid during the greater part of their life cycle and meiosis was assumed to be post-zygotic, however without direct evidence (Garnham 1966, Sinden, 1978; Walliker, 1983; Kemp et al., 1985; Casaglia et al., 1985a). Very recently the exact time of meiosis has been determined in P. berghei: within 2.5 hour after fertilization typical meiotic figures (condensed chromosomes, synaptonemal complexes) have been observed with the electron microscope in the nucleus of the zygote (Sinden et al., 1985; Sinden and Hartley, 1985). This observation makes earlier conclusions on the time of meiosis (in the young oocyst), based on cytological studies (Bano, 1959; Canning and Anwar, 1968, 1969) invalid. If meiosis in malaria parasites follows the normal eukaryotic pattern, i.e. if formation of synaptonemal complexes is preceded by genome duplication, normal patterns of recombination of genes may be expected.

3. The life cycle of mammalian malaria parasites

The parasite in the mammalian host

Tissue phase. A malaria infection in the mammalian host starts with the injection of sporozoites by a biting mosquito. These sporozoites circulate in the blood for a short period (30-60 min), then enter parenchymal cells of the liver and start a process of development and multiplication (exo-erythrocytic schizogony) resulting in many thousands of parasites, the tissue stage merozoites. Within the liver cell, sporozoites of some species can transform into resting stages, called hypnozoites which may enter the cycle of differentiation and multiplication after prolonged periods of rest.

Erythrocytic phase. Merozoites released from tissue schizonts into the blood circulation rapidly invade red blood cells. The newly invaded parasites, called ringforms, are small, rounded forms sometimes containing a vacuole displacing the cytoplasm to the periphery. These stages transform into feeding stages, known as erythrocytic trophozoites. During growth they consume the haemoglobin of the red blood cell, leaving an iron containing pigment as waist product.The trophozoite stage can undergo either asexual or sexual differentiation.

Asexual trophozoites multiply by a process of schizogony; the nucleus of the parasite divides - without subsequent cell fission - into a variable number of nuclei (8-32); the multinucleated mature schizont then starts to divide its cytoplasm producing a similar number of haploid daughter merozoites. Parasites showing mitotic activity are termed schizonts. After rupture of the red blood cell the free merozoites invade new red blood cells and schizogony is repeated.

Some trophozoites, however, do not continue to multiply asexually but differentiate within the red blood cell into uninucleate sexual cells, the gametocytes. In all Plasmodium species the female (macro-)gametocyte has a small, compact nucleus while the male (micro-)gametocyte has a larger, diffuse nucleus. Both sexes almost completely fill their host cell and contain many pigment granules.

The parasite in the mosquito host

Sexual reproduction takes place in female mosquitoes of the genus Anopheles. When a mosquito ingests Plasmodium infected blood, only the mature sexual cells undergo further development. This involves an escape from the host cell, a maturation process of the female gametocyte to form a spherical female gamete and a division of the microgametocyte's nucleus to form eight haploid male gametes. The formation and subsequent release of the thread-like male gametes is called exflagellation and takes about 10 minutes. Fertilization follows and the zygote transforms into a banana-shaped, motile stage (ookinete) which penetrates the midgut wall. The penetrated ookinete rounds up below the midgut epithelium. This stage, the oocyst, enlarges, the nucleus becomes highly polyploid and divides frequently, followed by multiple cell fissions to form thousands of sporozoites. After bursting of the oocyst wall, the free sporozoites migrate to and enter the salivary glands of the mosquito, waiting for being injected into a new mammalian host.

A diagram illustrating the life cycle of P. berghei and P. falciparum is shown in Fig. 1.

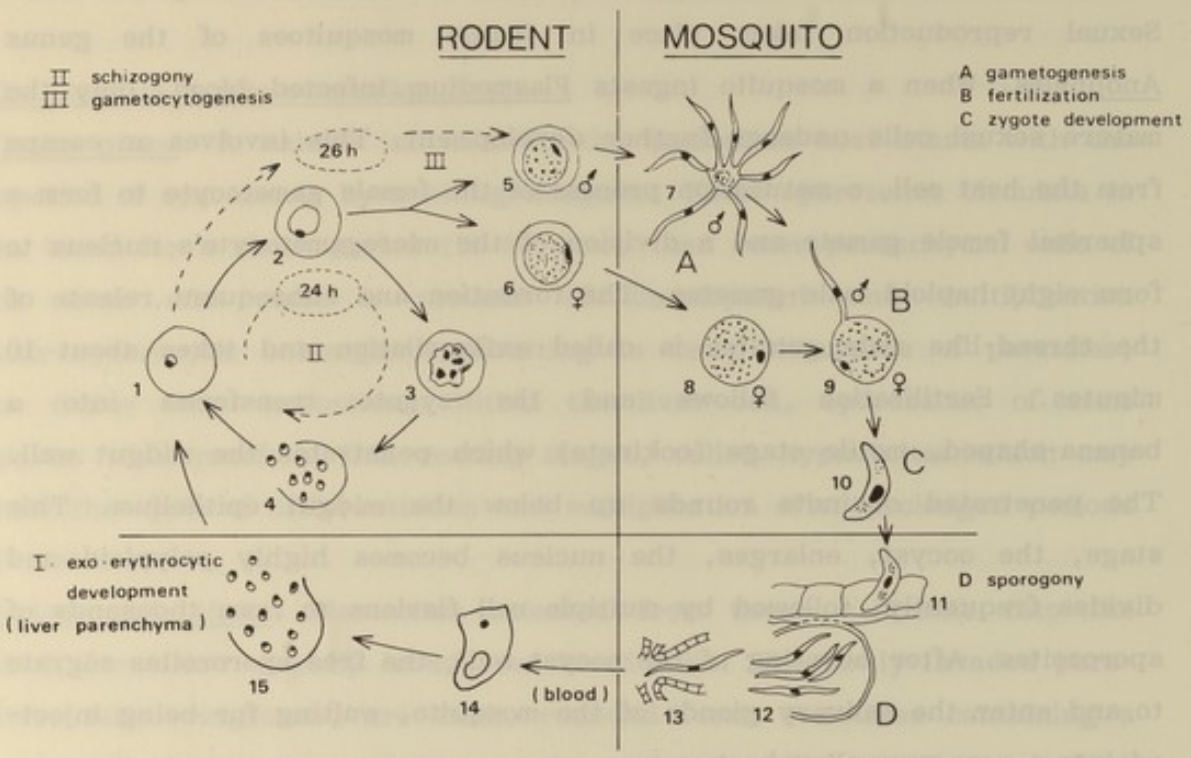

Fig. 1a. **Life cycle of the rodent malaria parasite Plasmodium berghei.**

1 - ringform
2 - trophozoite
3 - young schizont
4 - mature schizont with merozoites
5 - microgametocyte
6 - macrogametocyte
7 - exflagellating microgametocyte, producing 8 gametes
8 - macrogamete
9 - fertilization
10 - mature ookinete
11 - ookinete penetrating the midgut wall
12 - mature oocyst with sporozoites
13 - sporozoite in salivary gland
14 - parasite within liver parenchyma cell
15 - exo-erythrocytic schizont

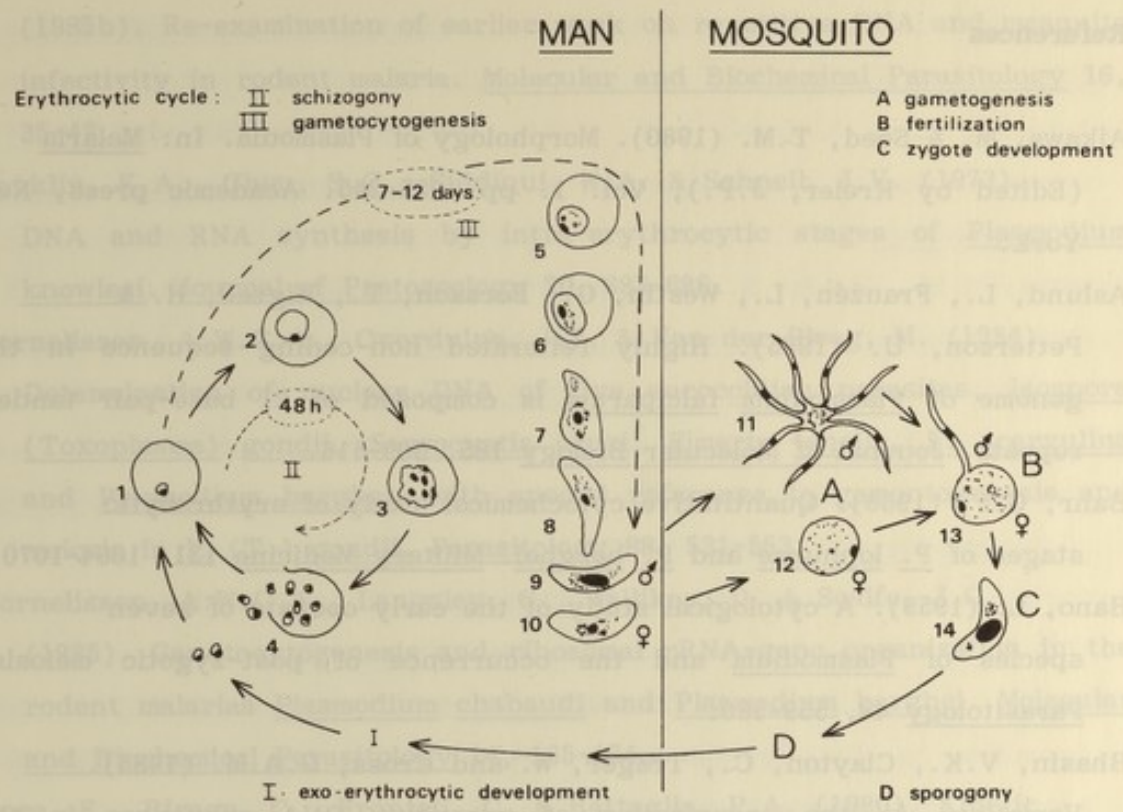

Fig. 1b. **Life cycle of the human malaria parasite Plasmodium falciparum.**

1 - ringform
2 - trophozoite
3 - young schizont
4 - mature schizont with merozoites
5 - gametocyte stage I
6 - stage II
7 - stage III
8 - stage IV
9 - mature microgametocyte
10 - mature macrogametocyte
11 - exflagellating microgametocyte, producing 8 gametes
12 - macrogamete
13 - fertilization
14 - mature ookinete

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Chapter II

IN VITRO CULTURE OF PLASMODIUM BERGHEI USING A NEW SUSPENSION SYSTEM

B. Mons, C.J. Janse, J.J.A.B. Croon and H.J. van der Kaay. 1983. International Journal for Parasitology, 13, 213-217.

Summary

Attempts were made to develop techniques for the continuous in vitro culture of Plasmodium berghei. The candle jar method (Trager & Jensen, 1976) proved to be insufficient for the culture of this rodent malaria parasite. Parasitaemia decreased rapidly after the first schizogonic cycle in culture. A simple suspension technique was developed using a newly designed culture apparatus which can be placed in the laminar-flow. All manipulations necessary for the refreshment of medium and cells can be made with almost no disturbance of the culture conditions. With this system it was possible to culture P. berghei repeatedly for more than a week, completing at least four schizogonic cycles with considerable merozoite invasion and a 2-6 fold multiplication. Infection rates of up to 6% were achieved and cultures were maintained for 9 days. Several specific properties of P. berghei and the differences between the candle jar method and the new suspension method are discussed to explain the results obtained in both systems.

Introduction

In 1976 Trager and Jensen were the first to describe a method for the continuous culture of the erythrocytic stages of the human malaria parasite Plasmodium falciparum. Several workers have tried to culture animal malaria parasites, as that would enable a comparison of results obtained from experiments in vitro with those obtained in vivo, which is not possible with human parasites. Apart from P. falciparum, prolonged in vitro culture has now been reported for three other primate malaria parasites, P. fragile by Chin, Moss & Collins (1979), P. knowlesi by Butcher (1979) and Wickham, Dennis & Mitchell (1980) and recently for P. cynomolgi by Nguyen-Dinh, Gardner, Campbell, Skinner & Collins (1981).

It would be very useful to be able to culture the rodent malaria parasites P. berghei, P. vinckei, P. chabaudi and P. yoelii in vitro. This would apply especially to P. berghei, as this species can be considered as an important research object, because it can easily be cyclically transmitted in the laboratory. Several attempts have been made to culture P. berghei (e.g. Tiner, 1969; Williams & Richards, 1973; Smalley & Butcher, 1975; Guru & Sen, 1978) and P. chabaudi (e.g. Geiman, Siddiqui & Schnell, 1966; Trigg, 1968). Culturing these parasites, however, proved to be extremely difficult. The investigators have only succeeded in maintaining short-term cultures of the intra-erythrocytic stages for one or two schizogonic cycles, with a decreasing parasitaemia. In the present report for the first time a simple technique for the in vitro culture of P. berghei achieving considerable merozoite invasion and rising parasitaemia during several subsequent cycles is described.

Materials and Methods

Medium. 10.41 g of RPMI 1640 powder (Flow) was dissolved in 960 ml deionized water to which 5.94 g Hepes was added. This solution was sterilized through a 0.2 µm Pall filter and stored at -20°C. The maximum

period of storage was 1 month. Just before use the medium was supplemented by adding 4.2 ml of a sterilized 5% $NaHCO_3$ solution, 11 ml fetal calf serum (FCS) (Boehringer, Mannheim) and 5000 i.u. neomycin to 96 ml of stock solution to give complete medium.

Blood. Infected erythrocytes were obtained by cardiac puncture under ether anaesthesia from Swiss mice (25-30 g) intraperitoneally (i.p.) infected with P. berghei ANKA, having a parasitaemia of 2-10%. The blood was suspended in an equal volume of complete medium with 20 i.u./ml heparin and centrifuged at 200 g for 10 min. Supernatant and the upper layer of cells, containing the buffy-coat and about 10% of the red cells were removed. This layer of cells included the majority of multiple infections and schizonts of P. berghei. The remaining erythrocytes were suspended in medium (approx. three times the volume of packed cells) and passed through a column of Whatman CF11 cellulose powder of 1 x 5 cm in order to remove the remaining leucocytes. Subsequently the erythrocyte suspension was diluted 1:4 with complete medium and centrifuged at 450 g for 10 min. All procedures were performed under sterile conditions at room temperature. Reticulocytosis was induced in Swiss mice (25-30 g) by one i.p. injection of 60 mg/kg body wt. of phenylhydrazine-HCl. Four to six days later the red blood cells (RBCr) of these mice were isolated following the same procedure as described for infected erythrocytes and used for dilution of cells in culture. We used the candle jar method as described by Trager & Jensen (1977) with some slight modifications; infected erythrocytes were mixed with RBCr in complete medium to give an initial parasitaemia of 0.5-2% and an 8-12% erythrocyte suspension. This mixture was dispensed in 35 mm plastic Petri dishes and placed in the candle jar at 37°C. Culture medium was changed twice a day. At 48-50 h intervals 20-50% of cells in culture was replaced by the same amount of uninfected cells (RBCr). For the candle jar these circumstances are referred to as standard conditions. Samples were taken from the culture material and the number of intra-cellular parasites per 4000-5000 red blood cells was counted from smears fixed in methanol and stained with Giemsa. Differential

parasite counts were made on 100 parasites. This was discontinued when the parasitaemia dropped below 0.5%.

Culture apparatus. A diagram of the culture apparatus designed for the suspension system is given in Fig. 1. The culture chamber has an inner diameter of 3.5 cm and a height of 2.5 cm. This apparatus can be placed in the laminar flow. All manipulations necessary for the refreshment of medium and cells can be made with hardly any disturbance of the culture conditions. In the culture chamber of this apparatus, the cells were kept in suspension by a magnetic stirrer (2.6 x 0.4 cm) at a speed of about 100 rev./min.

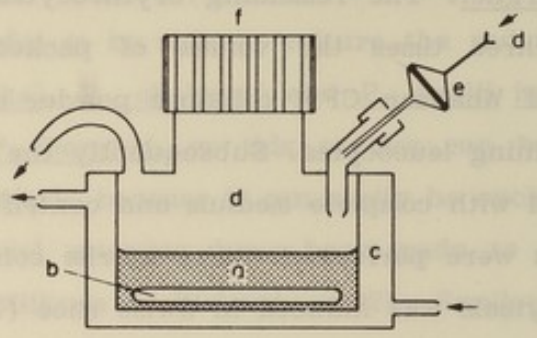


FIG. 1. A diagram of the suspension culture apparatus. a. erythrocyte suspension; b. magnetic stirrer; c. glass water jacket; d. gas mixture; e. 0·2 μm membrane filter; f. screw cap.

A continuous stream of a filter-sterilized gas mixture of 5% CO_2, 10% O_2 and 85% N_2 was led through the culture compartment. Temperature was kept constant at 37°C, except for the first 20 h when temperature was kept at 30°C. This has been introduced to allow for a better timing of the replacement of the culture medium and the addition of fresh RBCr. Infected erythrocytes (0.15-0.5 ml packed cells) were added to 5 ml of complete medium in the culture chamber. Twenty hours later RBCr were added to give a parasitaemia of 0.5-2% and a 8-12% cell suspension. The temperature was then raised to 37°C. Twice a day the medium was changed manually with a Pasteur pipette. Two hours earlier the magnetic stirrer was automatically switched off, and the cells sedimented on the bottom of the

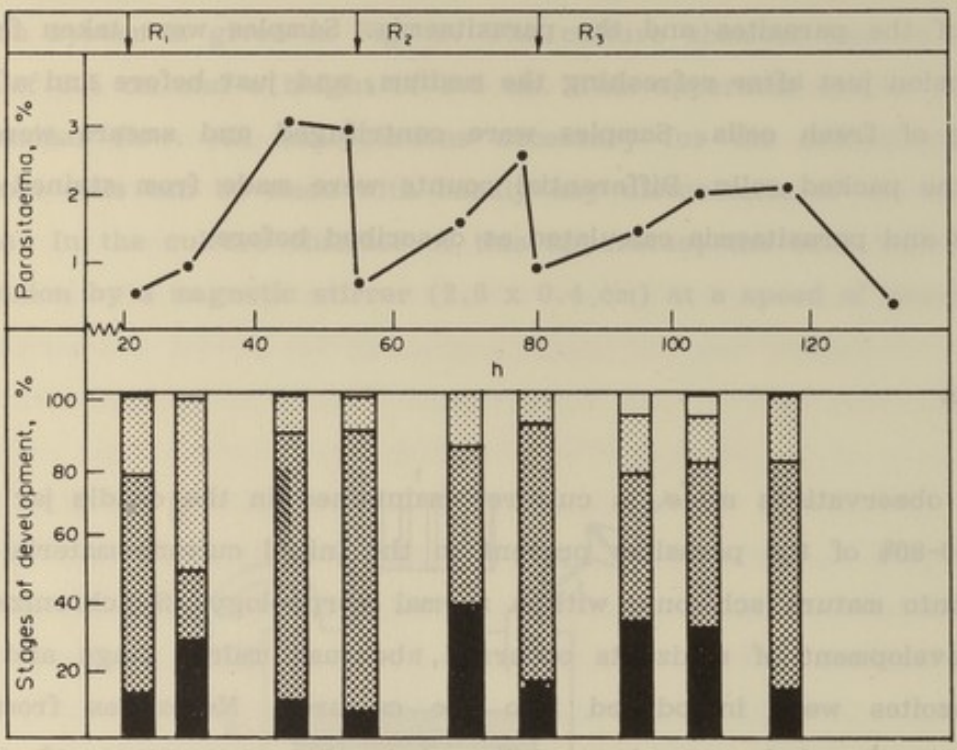
compartment. For the suspension cultures, these circumstances are referred to as standard conditions. The moment of adding RBCr and their quantity during the culture period was dependent on the stage of development of the parasites and the parasitaemia. Samples were taken from the suspension just after refreshing the medium, and just before and after the adding of fresh cells. Samples were centrifuged and smears were made from the packed cells. Differential counts were made from stained Giemsa smears and parasitaemia calculated as described before.

Results

Initial observations made on cultures maintained in the candle jar showed that 50-80% of the parasites present in the initial culture material developed into mature schizonts with a normal morphology. Synchronization of the development of schizonts occurred, because mainly rings and young trophozoites were introduced into the cultures. Merozoites from these schizonts invaded new host cells, but the percentage of infected erythrocytes dropped after 30 h of culture. An increase of the number of extracellular and degenerated forms was observed. From day 0 to 6 parasitaemia decreased gradually to levels below 0.05% on day 5. In the candle jar, as in the suspension cultures, schizogony took 25-30h at 37°C (Figs. 2, 3).

When the cultures were not provided with fresh RBCr, a more marked decrease of parasitaemia was observed after the first cycle and already within 3-4 days the percentage of infected cells decreased to levels below 0.05%.

At a culture temperature of 28 instead of 37°C, the growth of rings into schizonts took 50-70 h in our experiments (Table 1), while the parasitaemia decreased to 0.05% within 8-9 days. No better results were obtained using medium 199 (Difco), rat serum, a higher concentration (15%) of FCS or an extra addition of nutrients: glucose (2 g/l), histidine (10 mg/l) and of ATP (10 mg/l) to complete medium.



Legends to Figs. 2 *and* 3: —•— = parasitaemia, □ = not differentiated, ▨ = schizonts, ▩ = trophozoites, ■ = rings, R = reinvasion, ↓ = adding fresh RBCr, h = hours.

Fig. 2. The course of parasitaemia (%) and the changes in the ratio of the different developmental stages (% of total) of the parasites in a typical culture experiment with *P. berghei* in the suspension culture apparatus, showing three complete schizogonic cycles during 130 h in culture.

In the suspension system the development of the parasite during the first cycle was slowed down by maintaining the culture during the first 20 h at 30 instead of 37°C. The morphology of the parasites and synchronization of schizogony during the first cycle did not significantly differ from that obtained in the candle jar. However, the percentage of merozoites invading new host cells was much higher in the suspension apparatus, resulting in a 2-6 fold multiplication (Figs. 2, 3). These newly invaded merozoites developed via morphological normal rings and trophozoites into "second generation" schizonts. In this manner 4-5 subsequent cycles could be completed (Fig. 3).

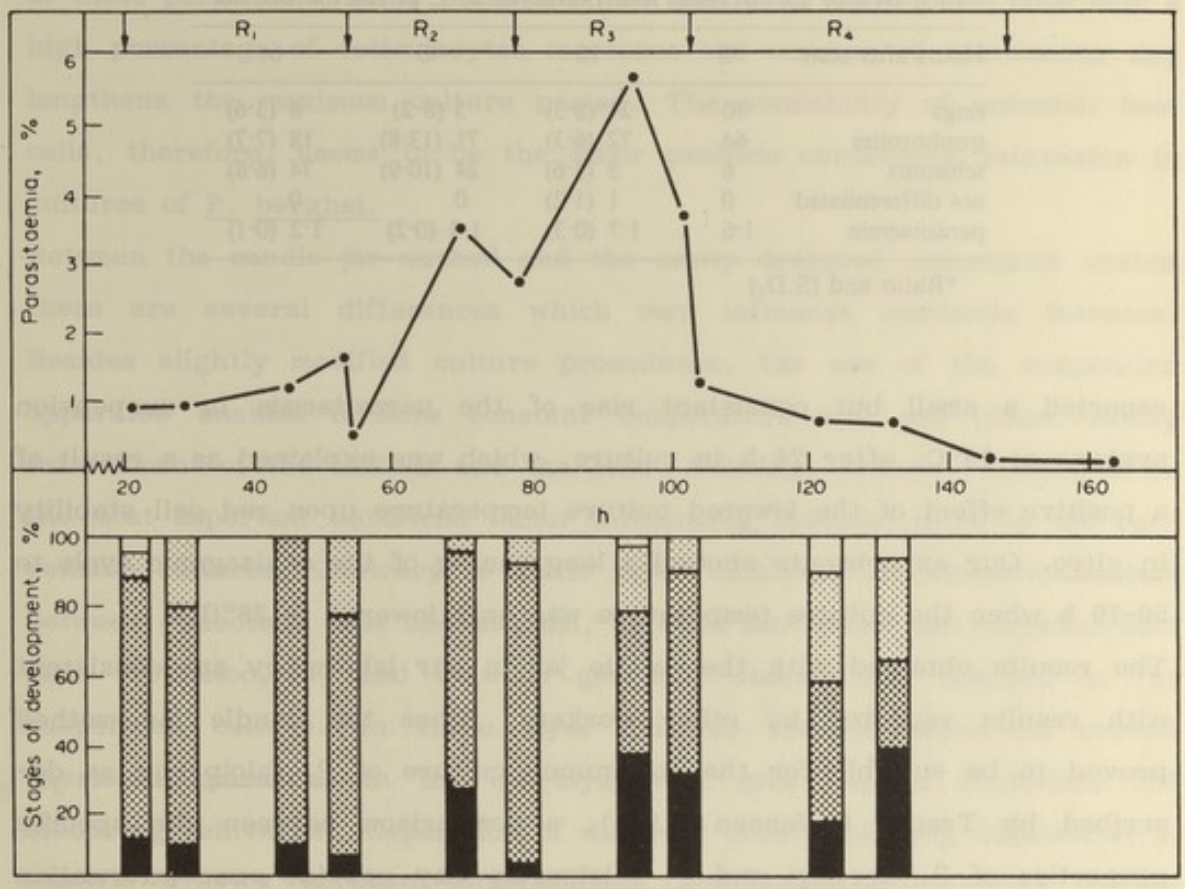


FIG. 3. The course of parasitaemia (%) and the changes in the ratio of the different developmental stages (% of total) of the parasites in a typical culture experiment with *P. berghei* in the suspension culture apparatus, showing four complete schizogonic cycles during 160 h in culture.

When the culture period exceeded 100 h usually parasitaemia dropped to levels below 0.05% and the cultures were terminated.

Discussion

So far, there are no reports on the successful culture of P. berghei for more than one complete erythrocytic cycle in vitro. Although parasites developed into schizonts, most of the workers did not report merozoite invasion. In general, parasitaemia decreased rapidly and the amount of degenerated material increased in the cultures. Smalley & Butcher (1975)

TABLE 1—MEAN PARASITAEMIA (%) AND CHANGES OF THE RATIO OF DIFFERENT DEVELOPMENTAL STAGES (% OF TOTAL) OF *P. berghei* DURING THE FIRST 64 h OF SIX CULTURES MAINTAINED AT 28°C IN THE CANDLE JAR

Hours after start	0	16	40	64
rings	30	24*(8·3)	5 (8·2)	8 (3·6)
trophozoites	64	72 (6·3)	71 (13·8)	18 (7·7)
schizonts	6	3 (5·6)	24 (10·9)	74 (6·8)
not differentiated	0	1 (1·2)	0	0
parasitaemia	1·6	1·7 (0·3)	1·3 (0·2)	1·2 (0·1)

*Ratio and (S.D.)

reported a small but consistent rise of the parasitaemia in suspension systems at 15°C, after 24 h in culture, which was explained as a result of a positive effect of the lowered culture temperature upon red cell stability in vitro. Our experiments showed a lengthening of the schizogonic cycle to 50-70 h when the culture temperature was only lowered to 28°C.

The results obtained with the candle jar in our laboratory are consistent with results reported by other workers. Since the candle jar method proved to be suitable for the continuous culture of P. falciparum as described by Trager & Jensen (1976), a comparison between the specific properties of P. berghei and P. falciparum may provide some information about the possible causes of the failures with the candle jar method for the former species. The schizogonic cycle of P. falciparum takes approx. 48 h, while P. berghei completes schizogony within 24 h. The latter, therefore, may have a more intensive metabolism, which means a faster exhaustion of the culture medium and accumulation of metabolites. In contrast to P. falciparum, P. berghei shows a striking preference for reticulocytes (Garnham, 1980). In our cultures the proportion of red cells recognizable by Giemsa staining as reticulocytes never exceeded 30%. This may be a limiting factor for the reinvasion of P. berghei merozoites in static-layer cultures such as the candle jar method. We obtained no evidence from our experiments with the candle jar that the culture medium and the gas mixture used were limiting factors for the intra-erythrocytic growth of P. berghei in vitro. Under the given circumstances parasites develop from ring-stages into morphologically normal schizonts. Merozoites from these

schizonts are capable of reinvading reticulocytes, but a very small number of these parasites actually invades a new host cell. Adding red cells with a high percentage of reticulocytes increases the number of reinvasions and lengthens the maximum culture period. The availability of potential host cells, therefore, seems to be the major problem concerning reinvasion in cultures of P. berghei.

Between the candle jar method and the newly designed suspension system there are several differences which may influence merozoite invasion. Besides slightly modified culture procedures, the use of the suspension apparatus enables a more constant temperature and gas phase during refreshment of the medium and the cells. Stirring, however, seems to be the most important beneficial factor influencing invasion rates in the suspension cultures. Contrary to static layer cultures, an intensive contact between (infected) cells and medium, between merozoites and potential host cells (reticulocytes) and between gas and medium was realized in the suspension culture. In static layer cultures micro-environments around developing parasites in the cell-layer may give rise to conditions not favouring reinvasion. Separation of different cells caused by differences in specific gravity between infected and uninfected erythrocytes during settling of cells may also play an important role in static layer cultures. Finally, mechanical forces (stirring) may be important as an induction for the rupture of mature schizonts, comparable to the pressure that schizonts undergo during their passage through the capillaries of the mammal host in the natural situation. In our experiment stirring appears to be of the highest importance for the prolonged in vitro culture of P. berghei. This aspect and frequent addition of fresh reticulocytes may also be the key for the culture of other vivax-type malaria parasites. The sudden fall of parasitaemia after one week is not yet clarified. Degeneration of erythrocytes present from the very beginning of the culture may play a role. Further development of the culture techniques may result in a continuous culture of P. berghei.

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Chapter III

LONG TERM IN VITRO CULTURES OF PLASMODIUM BERGHEI AND PRELIMINARY OBSERVATIONS ON GAMETOCYTOGENESIS

C.J. Janse, B. Mons, J.J.A.B. Croon and H.J. van der Kaay. 1984. International Journal for Parasitology, 14, 317-320.

Summary

Several long-term in vitro cultures of the rodent malaria parasite Plasmodium berghei were established. In these cultures, ranging over 17-90 days, peak parasitaemias of over 20% and multiplication rates of up to 7.7 were observed. A previously described culture method was used. The method for medium refreshment was changed and rat erythrocytes were used as host cells. The long-term cultivation of Plasmodium berghei enables us to study the process of gametocytogenesis since male and female gametocytes were produced in all cultures and reached full maturity, demonstrated by exflagellation and fertilization in vitro.

Introduction

In vitro cultures of the erythrocytic stages of Plasmodium species provide valuable experimental tools for several aspects of malaria research. Long-term in vitro cultures are reported for some primate malaria parasites (for a review see Trager, 1982). Recently we reported results with a new culture system used to maintain the rodent malaria parasite Plasmodium berghei in culture for up to 9 days (Mons, Janse, Croon & Van der Kaay, 1983).

P. berghei in rodent hosts is widely used as a laboratory model for the investigation of several aspects of the life cycle of Plasmodium, both in rodent and mosquito hosts. In this system the in vitro culture of the erythrocytic stages of the parasite offers the possibility to compare results from in vitro experiments with results obtained in vivo. We are particularly interested in the development of gametocytes of P. berghei. In vitro experiments could benefit the investigation of the poorly understood process of gametocytogenesis (Sinden, 1983). In this paper we give a method for the establishment of long-term cultures of P. berghei and preliminary observations on the production of gametocytes in these cultures.

Materials and Methods

Basically the cultivation procedure as described in detail by Mons et al. (1983) was followed. Some modifications which enabled a more prolonged cultivation of the parasite are described here.

Medium. For all cultures RPMI 1640 (Flow), with Hepes (5.94 g/l), and $NaHCO_3$ (42 ml/l of a 5% solution) was used as described by Trager & Jensen (1976), supplemented with fetal calf serum (FCS) (100 ml/l) and neomycin (50000 i.u./l) to give complete medium.

Blood. Erythrocytes from young (6-8 weeks) Wistar rats were used as host cells. Parasitized erythrocytes were collected by cardiac puncture from

rats infected i.p. with P. berghei (ANKA-strain), having a parasitaemia of 1-4%. For dilution of the cultures, fresh red blood cells with a relatively high proportion of reticulocytes (RBCr) were obtained by cardiac puncture from rats 4-6 days after treatment with phenylhydrazine-HCl (60 mg/kg body wt.). Leucocytes were removed using Whatman CF11 cellulose powder. The RBCr were stored up to 7 days at 4°C in complete medium which was refreshed every second day.

Culture procedure. Cultures were maintained in a culture apparatus developed by Mons et al. (1983), in which cells were kept in suspension by a magnetic stirrer. Culture temperature was 37°C except for the weekends when temperature was lowered to 32-34°C. At this temperature a less frequent replacement of the culture medium was possible, because of the reduced metabolism of the parasites. Cultures were initiated with cell suspensions of 4-6% and parasitaemias of 1-4%. Twenty hours after start when schizonts were present RBCr were added in order to reduce the parasitaemia to 0.5-1% and to give an 8-12% cell suspension. Thereafter dilution of cultures with RBCr was performed every day. Culture medium was changed manually twice a day, except for the weekends, when medium was changed once a day. Medium was refreshed in the following way: under sterile conditions the complete suspension (medium and cells) was removed from the apparatus and centrifuged (200 g, 5 min.). In the meantime fresh medium was brought to 37°C and gassed in the apparatus. After centrifugation supernatant was removed from the culture material and the packed cells were dispensed again in the fresh medium.

Assessment of parasite growth. Smears were made before and after adding of RBCr and stained with Giemsa. The parasitaemia was determined by counting the number of intra-cellular parasites per $4\text{-}5 \times 10^3$ erythrocytes. The multiplication rate was defined as the ratio of the parasitaemia just after adding RBCr and parasitaemia just before the next dilution, 24 h later. The average multiplication rate is calculated from multiplication rates of every day of culture except for the weekends. For the calculation of the percentage of sexual parasites only morphologically mature gametocytes were counted as young gametocytes are not easily identifiable. Gametocytes

were classified as "mature" when they nearly or completely filled their host cells and could be differentiated in male and female. With Giemsa staining the macrogametocyte's cytoplasm stains blue and the small nucleus red. The microgametocyte stains light yellow-grey with an extended nucleus of pink colour. Dark pigment granules are visible in the cytoplasm of both sexes. The number of gametocytes was counted per 2-5 x 10^2 parasitized erythrocytes.

Exflagellation of gametocytes from culture was stimulated as follows: a sample of the culture material was centrifuged (200 g, 5 min.). The sedimented cells were resuspended in an equal volume of RPMI 1640 with 10% FCS, adjusted to pH 7.8-8.1 with 5% $NaHCO_3$. One droplet of this suspension was placed on a microscope slide to observe exflagellation. Occasionally the remaining suspension was placed in Petri dishes at 19-22°C for fertilization and ookinete formation. After 20-24 h smears were made from this material, stained with Giemsa and examined for ookinetes. Infectivity of cultured parasites was regularly tested. The course of infection and gametocytogenesis was examined each time in three animals (mice or rats).

Results

In three cultures maintained for respectively 17, 41 and 90 days, parasites were growing and multiplying, showing a normal morphology. Parasitaemias and multiplication rates of three long-term cultures are shown in Table 1. The description of culture C is subdivided in three subsequent periods of 30 days.

The average percentage of infected cells just before the cultures were diluted ranged between 4.4 and 15%. Peak parasitaemias of over 20% were repeatedly observed in the second and third period of culture C. During the different culture periods multiplication rates reached maximum values of 4.6-7.7 with an average of 3.0-3.4.

To study the feasibility of starting subcultures, several subcultures were initiated and observed for 5-10 days. Initial parasitaemias of these cultures varied between 1 and 5%. Multiplication rates observed in the subcultures were in the same range as those of the original cultures. For dilution of cultures B and C (third period) the use of RBCr which were stored at 4°C for several days had no detectable inhibitory effect on the multiplication of the parasites. Cultures B and C were in good condition when terminated and parasites produced normal infections when they were injected into mice or rats.

TABLE 1—PARASITAEMIAS AND MULTIPLICATION RATES OF THREE LONG-TERM CULTURES OF *P. berghei*

Culture	Period (days)	Parasitaemia before dilution			Multiplication rate		
		Max.*	Mean	(S.D.)	Max.	Mean	(S.D.)
A	0–17†	7·0	4·7	(1·3)	5·7	3·2	(1·3)
B	0–41	15·5	10·8	(4·5)	4·6	3·0	(1·4)
C	0–30	9·0	4·4	(1·9)	7·7	3·1	(1·7)
	31–60	21·5	12·7	(4·9)	4·8	3·4	(1·0)
	61–90	22·0	15·0	(5·6)	5·2	3·4	(1·2)

*Max. = highest parasitaemia/multiplication rate observed during the culture period.

†Terminated after bacterial contamination.

Observations on the production and the maturation of gametocytes in culture are shown in Table 2. During all culture periods morphologically mature gametocytes of both sexes were produced in low but variable numbers. The morphology of these gametocytes corresponded with the known morphology in vivo. In general the percentage of gametocytes was below 1%. Occasionally percentages up to 6% were counted in cultures A and C (first period). Once or twice a week the capacity of the microgametocytes to exflagellate was tested. From these tests it appeared that even after 58 days in culture C fertile microgametocytes were present. In the third period of culture C no exflagellation could be demonstrated. Parasites, however, had not lost their capability to produce mature gametocytes since parasites injected into rodents during this period produced morphologically

TABLE 2—GAMETOCYTOGENESIS IN CULTURES OF *P. berghei* AND IN RODENTS INFECTED WITH CULTURED PARASITES

Culture	Period (days)	Gametocytes (% of infected cells)	Last day exflagellation was observed	Gametocytogenesis in rodents infected with culture material			
		Range (%)		Infected on day	Infection	Mature gametocytes	Exflagellation
A	0–17	1–6	14	10	+	+	+
				17	+	+	+
B	0–41	0–1	35	29	+	+	+
				40	+	+	+
C	0–30	1–6		64	+	+	+
	31–60	0–2	58	71	+	+	+
	61–90	0–1		85	+	+	+
				90	+	+	+

mature gametocytes and microgametocytes from tailblood were able to exflagellate. To test the fertility of both male and female gametocytes produced in culture C (second and third period) attempts were made to perform fertilization and ookinete formation in vitro. Ookinetes developed on day 41 of culture, showing a morphology comparable to that of ookinetes in smears from mosquito midguts 24 h after feeding on infected rodents.

Discussion

In a previous paper (Mons et al., 1983) we reported considerable merozoite invasion and rising parasitaemia during the first days in cultures of P. berghei. After several days parasitaemia declined rapidly. In comparison with the earlier experiments there are two major differences in culture procedures which can account for the better growth of the parasites in the cultures described here: the use of rat erythrocytes as host cells and the introduction of centrifugation for replacement of the culture medium. In consequence of this changed method the sedimentation period of 2 h before medium refreshment was not necessary, shortening the period of disturbance to only 10 min. Since suspension of cells realized by stirring is

thought to be essential for the successful culture of P. berghei this can be considered as an advantage of the new method. With this method, however, we were not able to maintain two cultures for longer than 9 days when mouse RBCr were used as host cells. It appears therefore that the main reason for the prolongation of the culture is the use of rat erythrocytes as host cells. Red blood cells, particularly reticulocytes, of rats are more frequently multiple-infected than mouse erythrocytes (in vivo as well as in vitro). In rats up to 15 parasites per reticulocyte were sometimes observed, indicating that certain developmental stages of rat erythrocytes are extremely easy to invade by merozoites of P. berghei. Besides, the average number of merozoites in mature schizonts is higher in rat erythrocytes than in mouse cells, which can bring about a higher multiplication rate. It is well known that red blood cells undergo several degradative changes and become unstable in culture (Trigg, 1968; Trigg & Shakespeare, 1976). It might be that rat erythrocytes are more stable and mature less rapidly than mouse erythrocytes in vitro. This would be favorable for the culture since P. berghei invades mainly the younger stages of the erythrocyte.

The maintenance of P. berghei for longer periods in vitro is now possible. Our cultures meet the requirements for being useful in malaria research. High numbers of parasites can be obtained, cultures can be partly synchronized and the culture system is easy to handle. A possible disadvantage of P. berghei is the need of daily addition of reticulocytes. This is probably correlated to the rapid maturation and/or degeneration of rodent erythrocytes in vitro, since several workers report less frequent addition of fresh cells in cultures of primate vivax-type malaria parasites (Nguyen-Dinh, Gardner, Campbell, Skinner & Collins, 1981; Renapurkar, Pradhan, Sutar Shirodkar, Sharma, Ajgaonkar & Marathe, 1983).

An advantage of a P. berghei culture is that experiments in vitro can easily be combined with in vivo experiments in laboratory rodents. We have developed this system mainly for the study of gametocytogenesis. This process is difficult to investigate in vivo due to the variable and uncontrollable environment in the host and the asynchronic course of the

infection. Preliminary observations on the production of gametocytes in our cultures showed that morphologically mature gametocytes were present during all periods of culture in low numbers. It was not necessary to manipulate the culture conditions to induce gametocytogenesis. Although ookinete formation was not consistently obtained using cultured gametocytes, frequent failure of attempts to obtain ookinetes in vitro using material collected from infected rodents indicate that the techniques used need further optimization. Thus, absence of ookinetes did not mean absence of fertile micro- and macrogametocytes in culture.

The factors governing the induction and maturation of sexual forms in Plasmodium are not clear. Gametocytogenesis involves a genetic component but is also modulated by environmental factors (Carter & Miller, 1979; Mons & Van der Kaay, 1980; Smalley & Brown, 1981; Smalley, Brown & Bassett, 1981; Brockelman, 1982). Maturation of both male and female gametocytes was evidently completed in our cultures. We are now in the process of studying gametocytogenesis both in vivo and in vitro.

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Chapter IV

SYNCHRONIZED ERYTHROCYTIC SCHIZOGONY AND GAMETOCYTOGENESIS OF PLASMODIUM BERGHEI IN VIVO AND IN VITRO

B. Mons, C.J. Janse, E.G. Boorsma and H.J. van der Kaay. 1985. Parasitology, 91, 423-430.

Summary

Both asexual and sexual development of Plasmodium berghei were synchronized without chemical intervention using in vitro culture techniques. Combined in vivo and in vitro experiments were performed on the relationship between age, morphology and maturity of gametocytes. Schizogony took 22-23 hours in the experiments. At 26 hours post invasion (p.i.) the first males became capable of exflagellation. By 20 h p.i. the first gametocytes were recognizable in Giemsa stained smears but the sex was hardly distinguishable until maturity (26 h p.i.). Survival time of gametocytes was estimated at 26 hours in vitro and the same survival time was suggested for gametocytes in vivo. Schizonts of P. berghei apparently disappeared from the peripheral circulation upon maturity, rupturing almost immediately. Mature schizonts in vitro persisted up to 48 hpi in non-agitated cultures. No evidence was collected for sequestration of any sub-population of gametocytes.

Introduction

For most Plasmodium species in vitro experiments, which exclude uncontrollable physiological factors of the mammalian host, are difficult to combine with in vivo studies. Therefore, the use of the rodent malaria parasite Plasmodium berghei in the study of gametocytogenesis has advantages. Routinely performed cyclical transmission in the laboratory prevents loss of optimal and consistent gametocyte production (Yoeli & Most, 1960). Combined in vivo and in vitro experiments are feasible (Mons, Janse, Croon & Van der Kaay, 1983; Janse, Mons, Croon & Van der Kaay, 1984) and the vivax-type gametocytes of this species are expected to have a relatively short developmental time (Killick-Kendrick & Warren, 1968).
Working with the P. berghei-rodent system, however, has a major disadvantage in the asynchronous course of experimental infections which has prevented definitive experiments on the relationship between morphology, age and maturity of P. berghei gametocytes, experiments which were possible in P. falciparum (Smalley & Sinden, 1977). We were able to overcome these problems by developing techniques for the synchronization of erythrocytic schizogony and gametocytogenesis of P. berghei in vivo and in vitro.

Materials and Methods

Parasite, vector and vertebrate hosts

The ANKA strain of Plasmodium berghei was maintained in TBGif mice and transmitted cyclically using Anopheles atroparvus. Mechanical transmission was performed once a week using Swiss or TBGif mice. Parasites from the 1st or 2nd mechanical passage were used for the experiments.

Cultures

Infected cells were prepared for culture following the procedure described by Janse et al. (1984), omitting the passage of cells through CF 11

cellulose powder. The packed cells were incubated in 500 ml Erlenmeyer flasks. The flasks were gassed with a mixture of 10% O_2, 5% CO_2 and 85% N_2 for 5 min (200 cm^3/min) and placed on a shaker at a temperature of 37 ± 1°C.

After 20-23 h nearly all parasites had developed into schizonts without releasing their merozoites. The material was then removed from the flask, centrifuged (100 g, 5 min) and the upper 20% of the pellet, containing approximately 90% of the schizonts, was collected.

Synchronized infections

Synchronized infections were obtained by i.v. inoculation of ca. 10^8 purified schizonts/young Wistar rat (8-10 weeks old) treated with phenylhydrazine-HCl (120 mg/kg body wt.), 6 days prior to use.

Synchronized in vitro cultures

At 8-10 h post inoculation (p.i.) of the purified schizonts approximately 0.5 ml of tailblood from the rats was collected in complete culture medium, supplemented with 20 i.u./ml heparin. The cells were sedimented (200 g, 5 min.), resuspended in complete culture medium (8-10% (v/v), packed cells/medium) and incubated in a suspension culture apparatus (Mons et al., 1983), at a temperature of 37,5 ± 0.1°C. Cultures were not provided with fresh erythrocytes and stirring was stopped before 22 h p.i. reducing reinvasion to a minimal level. Medium was replaced twice a day.

Establishment of parasite development and exflagellation.

Thin bloodfilms were made from tailblood of the animals at 1-2 hour intervals and examined for parasites (Janse, Mons, Rouwenhorst, Van der Klooster, Overdulve & Van der Kaay, 1985). At the same time 5 µl of tailblood was tested for exflagellation. Simultaneously samples were taken from the cultures.

Differential parasite counts were made in Giemsa stained bloodfilms in 5 x 10^3 erythrocytes. All uninucleated parasites were counted as trophozoites

except for morphologically mature gametocytes, which were distinguished as male or female. Multinucleated parasites were counted as schizonts.

Results

Asexual parasites in vivo

The characteristic course of infection in rats during the first 50 h post-inoculation of purified schizonts was as follows. Parasitaemia increased rapidly and stabilized at approximately 1% after 4-6 h p.i. (Figs 1a and 2c). Up to 22 h p.i. overall parasitaemia remained at that level, followed by a sudden increase in parasitaemia at 22-23 h p.i. (Fig. 2c). At 20 h p.i. the first mitotic activity occurred (Fig. 1b). The number of distinct chromatin dots/parasite increased rapidly between 20 and 23h p.i.

The exact mean number of merozoites/mature schizont was hard to establish in vivo because in our infections nearly all schizonts disappeared from the peripheral blood at maturity (Fig. 1b) presumably sequestering in the capillaries of the inner organs. Between 26 and 28 h p.i. parasitaemia again reached a steady level but at 9.0-11.3% (Fig. 2c, Table 1). At 44-45 h p.i. parasitaemia increased again. At this time of infection the majority of reticulocytes became multiply-infected (2-10 parasites/cell) while mature erythrocytes were still uninfected. Multiplication rates in the 1st and 2nd cycle in different rats ranged between 8.9 and 10.5/cycle (Table 1).

Asexual parasites in vitro

In the parallel cultures, initiated at 8-10 h p.i., parasitaemia was comparable to that in vivo until 20 h p.i. Thereafter, some notable differences occurred. Large amounts of morphologically mature schizonts were present from 23 h p.i. onwards but only very few newly invaded parasites were seen. Mature schizonts were characterized by 12-16 distinct merozoites and a dark brown-yellowish dot of clustered pigment. The lack of stirring and suitable host cells after 24 h p.i. resulted in strikingly low multiplication rates.

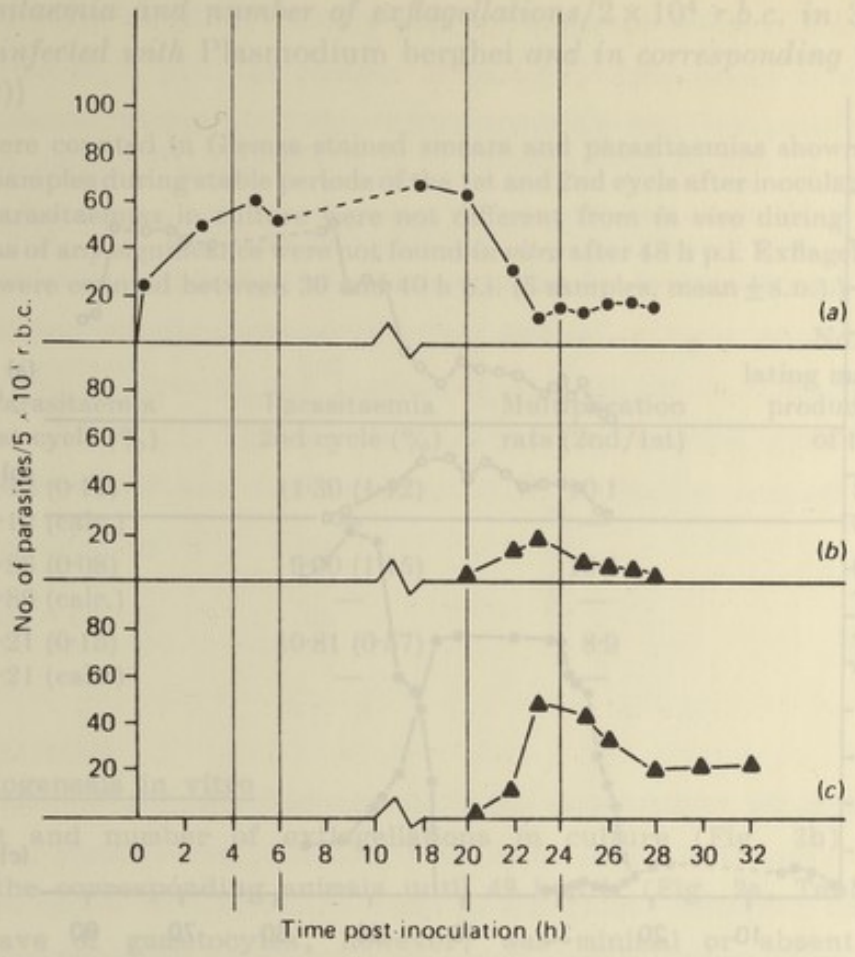

Fig. 1. Number of parasites from the 1st cycle in rat R, synchronously infected with *Plasmodium berghei* and in the corresponding *in vitro* culture R(c) during 32 h post-inoculation (p.i.). (*a*) Uninucleate parasites *in vivo*. Parasites remaining uninucleate after 23 h p.i. almost exclusively developed into gametocytes. Therefore at 28 h p.i. counts were terminated. Parasitaemias *in vivo* and *in vitro* were equal during the 1st cycle. Therefore the *in vitro* data are not shown. (*b*) Multinucleate parasites *in vivo*. Maturing schizonts disappeared rapidly from the peripheral circulation and were very short-lived. (*c*) Multinucleate parasites *in vitro*. Persistence of mature schizonts in the non-agitated culture resulted in a higher count than *in vivo*.

Gametocytogenesis in vivo

Gametocytes were produced in variable numbers during the infections. Up to 25 h p.i. no exflagellations could be demonstrated. Between 25 and 27 h p.i. exflagellation started, representing the first mature microgametocytes. The number of exflagellations stabilized at 28-33 h p.i. The pattern of exflagellation during infection (Fig. 2a) suggested that the development of gametocytes was also synchronized.

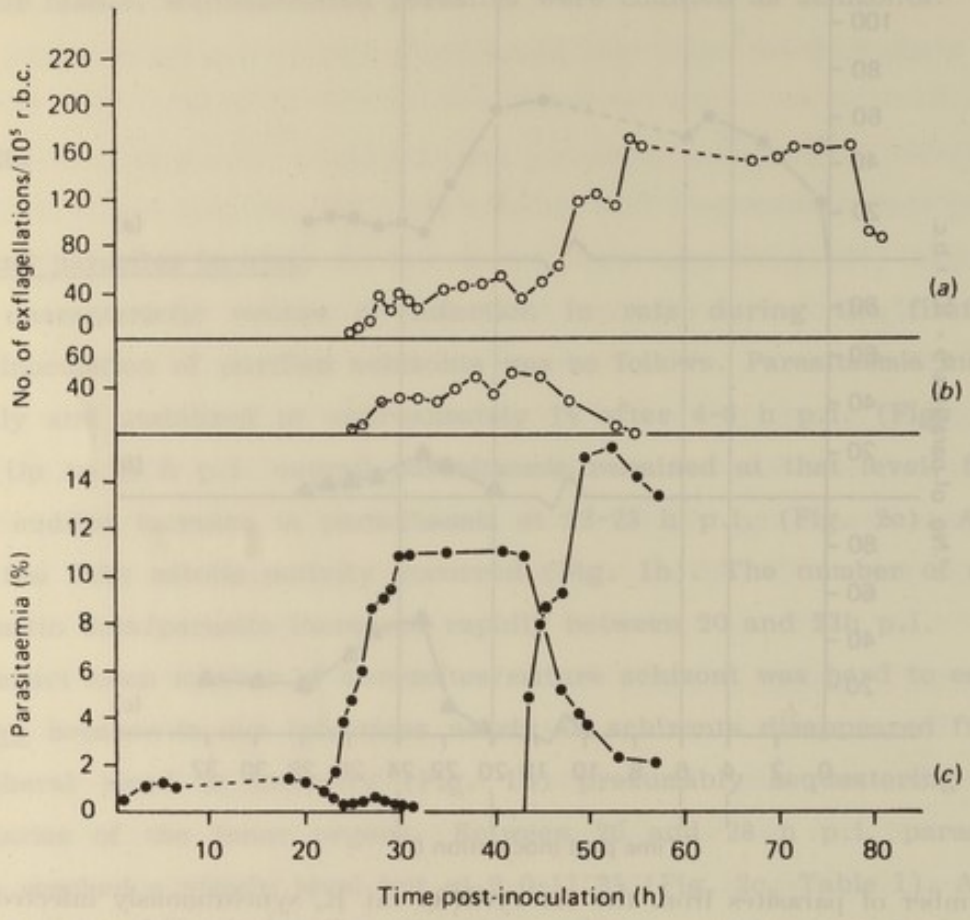

Fig. 2. Development of microgametocytes in relation to parasitaemia in rat R synchronously infected with *Plasmodium berghei* and in the corresponding *in vitro* culture R(c). (*a*) Number of exflagellating microgametocytes *in vivo* during 80 h post-inoculation (p.i.) (*b*) Number of exflagellating microgametocytes *in vitro* during 70 h after collection of infected tailblood from rat (R) at 10 h p.i. (*c*) Parasitaemia in rat R during 58 h p.i. Differential counts were made for parasites of each subsequent schizogonic cycle.

Between 48 and 49 h p.i. (Fig 2a) the number of exflagellations in vivo increased again, indicating maturity of microgametocytes developed from merozoites invaded around 22-23 h p.i. We therefore estimated the developmental time of gametocytes in our synchronized infections at approximately 26 h (Table 2).

Table 1. *Parasitaemia and number of exflagellations/2 × 10⁴ r.b.c. in 3 rats (P, Q, R) synchronously infected with* Plasmodium berghei *and in corresponding* in vitro *cultures (P(c), Q(c), R(c))*

(Parasites were counted in Giemsa-stained smears and parasitaemias shown as the mean (± S.D.) of 5 samples during stable periods of the 1st and 2nd cycle after inoculation of purified schizonts. Parasitaemias in culture were not different from *in vivo* during the 1st cycle, parasitaemias of any significance were not found *in vitro* after 48 h p.i. Exflagellations *in vivo* and *in vitro* were counted between 30 and 40 h p.i. (5 samples, mean ± S.D.).)

Rat (culture)	Parasitaemia 1st cycle (%)	Parasitaemia 2nd cycle (%)	Multiplication rate (2nd/1st)	No. of exflagellating males/2 × 10⁴ r.b.c. produced by parasites of the 1st cycle
P	1·12 (0·11)	11·30 (1·12)	10·1	8·06 (4·98)
P(c)	1·12 (calc.)	—	—	10·46 (4·60)
Q	0·86 (0·08)	9·00 (1·15)	10·5	9·10 (0·84)
Q(c)	0·86 (calc.)	—	—	7·60 (2·29)
R	1·21 (0·15)	10·81 (0·57)	8·9	7·80 (2·26)
R(c)	1·21 (calc.)	—	—	7·74 (2·18)

Gametocytogenesis in vitro

The onset and number of exflagellations in culture (Fig. 2b) paralleled those in the corresponding animals until 49 h p.i. (Fig. 2a, Table 1). The second wave of gametocytes, however, was minimal or absent in vitro (because of the lack of reinvasion described above). Thus, we conclude that the developmental time of gametocytes in vitro was comparable to that in vivo. Exflagellation in culture was stable between 28 and 50 h p.i. Thereafter the number of exflagellating males rapidly declined and decreased to zero at around 54 h p.i. (Fig. 2b).

Gametocyte morphology in vivo and in vitro

Since developing gametocytes of P. berghei are difficult to distinguish from asexual parasites of the same age we can only describe the morphology of gametocytes older than 22 h p.i. (when all asexual parasites were showing mitotic activity).

The young gametocytes did not completely occupy their host cell. The pigment granules of parasites considered as young gametocytes seemed to be more randomly distributed over the cytoplasm which stained faintly.

Table 2. *Developmental time of (micro-)gametocytes in 3 synchronously infected rats (P, Q, R) and in corresponding* in vitro *cultures (P(c), Q(c), R(c))*

(Duration of development was estimated by testing for the first capacitated microgametocytes. Developmental time of '2nd-wave' gametocytes was estimated by calculating the time difference between the onset of exflagellation and the onset of reinvasion around 22–23 h p.i.)

Rat (culture)	Onset of 1st exflagellation period (h p.i.)	Onset of 2nd exflagellation period (h p.i.)	Duration of 2nd gametocyte development (h)
P	26	49	49 − 23 = 26
P(c)	27	—	—
Q	26	48	48 − 22 = 26
Q(c)	27	—	—
R	25	49	49 − 23 = 26
R(c)	25	—	—

After 24 h p.i., when nearly all schizonts had disappeared from the peripheral blood, the only parasites present were rings and large uninucleated parasites almost filling their host cell. The latter were interpreted as being young gametocytes. These parasites were characterized by a slightly enlarged nucleus often surrounded by a small pink areola.

By 26 h p.i., when the first males were already capable of exflagellation we were still unable beyond doubt to distinguish microscopically microgametocytes from macrogametocytes. In spite of extensive examination of the smears no gametocytes could be found resembling the typical morphological features of mature males (Garnham, 1966). However, some gametocytes showed slightly larger nuclei than others, with a more pronounced pink areola probably representing fully developed males.

At 36 h p.i. the majority of males showed the classic morphology. Macrogametocytes were characterized during the entire exflagellation period by a condensed excentric nucleus and blue staining cytoplasm.

As found in vivo we were unable to recognize gametocytes in the cultures before 20 h p.i. Thereafter, the numbers and morphology of gametocytes in vitro resembled those in vivo. In vitro the fate of gametocytes

originating from the first cycle could be observed without complications. Shortly after 50 h p.i. the number of exflagellations began to decline and finally ceased at 54 h p.i. (Fig. 2b), indicating that the survival time of microgametocytes in terms of persistence of the ability to exflagellate was approximately 26 h in our in vitro experiments.

We examined the morphology of gametocytes in the cultures until 56 h p.i. The first sign of degeneration in vitro seemed to be a migration of the pigment granules towards the borders of the cell which occurred in some cases around 45 h p.i. This was followed by vacuolization of the cytoplasm (Yoeli & Most, 1960) and disintegration of the nucleus at around 54 h p.i.

Discussion

Asexual parasites

We estimated the degree of synchronicity in development of P. berghei in our rats by establishing the length of the periods wherein parasitaemia increased. Synchronicity was not lost during (at least) 3 subsequent schizogonic cycles. We therefore consider it unlikely that mature schizonts with merozoites capable of reinvasion will persist for prolonged periods in vivo. The heavy multiple infection of reticulocytes (up to 10 parasites/r.b.c.) in the 3rd schizogonic cycle in vivo indicates that parasitaemia, not reinvasion per se, is strongly restricted by the availability of suitable host cells for P. berghei.

Our impression is that more than two parasites growing in the same erythrocyte will not reach maturity. Development of P. berghei after 46 h p.i. was therefore severely compromised by the degree of multiple infection.

Gametocytes

The establishment of maturity of P. berghei gametocytes is very complex. We chose the simple exflagellation test for demonstration of the presence of

fully developed (capacitated) microgametocytes but do not draw any conclusion about the fertility of the gametes.

Developing gametocytes of P. berghei are difficult to recognize in Giemsa stained bloodfilms (Ramakrishnan, 1954; Garnham, 1966).

An advantage of working with synchronized infections is the ability to determine the final percentage of simultaneously developed cells transformed into gametocytes. Even after examining slides retrospectively no gametocytes could be distinguished beyond doubt before 20 h p.i.. Though Vincke & Lips (1948) stated that young gametocytes are often vacuolated, we believe this to be a characteristic feature of asexual trophozoites because at around 20 h p.i. the majority of the parasites showed this feature.

As found in vivo, we were not able to distinguish young gametocytes from asexual parasites in vitro up to 20 h p.i., indicating that the failure to do so was not caused by absence of these cells in the peripheral blood in vivo as described for P. falciparum (Garnham, 1966). Exflagellation started at 26 h p.i. when only blue-staining gametocytes were present. In order to exclude the possibility that this might be the result of phenylhydrazine treatment of the rat (thus producing large amounts of relatively young reticulocytes appearing as large blue cells in Giemsa-stained smears) we infected some non-treated Wistar rats with synchronized material and observed the gametocytes around 26 h p.i. which showed exactly the same morphology in mature erythrocytes as found in phenylhydrazine induced reticulocytes. Furthermore we observed these "blue-staining" microgametocytes after they had been induced to undergo gametogenesis. At 6-10 minutes after activation we observed activated males with clearly blue stained cytoplasm in Giemsa stained smears.

We therefore believe that under our staining conditions young but capacitated male gametocytes of P. berghei are characterized by blue staining cytoplasm rather than by pink-greyish cytoplasm. This means that between 24 and 36 h p.i. young male gametocytes can be misinterpreted as being female gametocytes, thus making counts on the sex-ratio in asynchronous P. berghei infections hardly feasible.

The similarity in numbers of exflagellation in vivo and in corresponding in vitro cultures for prolonged periods indicated that in our synchronized infections of P. berghei mature gametocytes did not disappear from the peripheral blood into the capillary beds as had been suggested for the closely related species Plasmodium yoelii (Landau, Miltgen, Boulard, Chabaud & Baccam, 1979).

Degeneration of gametocytes in vivo was difficult to observe because gametocytes of the subsequent wave were present in the blood. However, between 50 and 56 h p.i. gametocytes morphologically resembling the cells in vitro described as degenerating gametocytes were obviously present. In addition the slight dip in the increase in number of exflagellations in rat R between 50 and 55 h p.i. (Fig. 2a) is most likely caused by the degeneration of microgametocytes originating from the first cycle. This is confirmed by the decline in exflagellation numbers at 78 h p.i. We interpreted this decrease as the result of degeneration of 2nd wave gametocytes which developed from merozoites invaded at ± 23 h p.i. into capacitated microgametocytes at 23 + 26 = 49 h p.i. The marked decrease will be due to the high percentage of multiple infections in the 3rd cycle that will not develop into mature gametocytes. Further studies on the optimization of this rodent malaria model are in progress.

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Chapter V

IN VITRO FORMATION OF OOKINETES AND FUNCTIONAL MATURITY OF PLASMODIUM BERGHEI GAMETOCYTES

C.J. Janse, B. Mons, R.J. Rouwenhorst, P.F.J. van der Klooster, J.P.Overdulve and H.J. van der Kaay. 1985.
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Summary

In vitro formation of Plasmodium berghei ookinetes was studied. Gametocytes produced in vivo were obtained from heart and tail blood of Swiss mice and from blood removed from mosquitoes directly after feeding on these mice. In vitro produced gametocytes were obtained from short-term cultures of the erythrocytic stages of P. berghei. Reproducible ookinete production was obtained in medium RPMI 1640, pH 7.8-8.0, using in vivo and in vitro produced gametocytes. The morphology of developmental stages of ookinetes and degenerate forms at the light microscope level is described. More ookinetes were produced in medium RPMI 1640 compared to MEM and ookinete yield - defined as the ratio between the number of in vitro produced ookinetes/10^5 erythrocytes and the number of exflagellations/10^5 erythrocytes in the infected blood - increased with lower erythrocyte densities in the cultures within the range of dilutions tested. A linear relationship existed between gametocytaemia and the number of ookinetes produced. The methods for in vitro ookinete formation and for estimating ookinete yields enabled us to study aspects of functional maturity of gametocytes independent of mosquitoes. The numbers of

exflagellating gametocytes and in vitro ookinete yields in tail blood corresponded with those in heart blood and blood ingested by mosquitoes, suggesting a random distribution of functionally mature gametocytes within the vertebrate host.

Introduction

In malaria parasites gametogenesis, fertilization and ookinete development occur within the midgut of vector mosquitoes. These processes are influenced by factors present in the ingested blood (reviewed by Carter & Gwadz, 1980) and by mosquito factors. The latter may have stimulating (Nijhout, 1979) or inhibiting effects (Gass, 1977; Gass & Yeates, 1979). Mosquito factors can be excluded by using techniques for in vitro fertilization and ookinete development. In vitro production of Plasmodium berghei ookinetes was reported by Weiss & Vanderberg (1977) and Chen, Seeley & Good (1977). The purpose of the present study was to obtain reproducible methods for in vitro ookinete formation using in vivo and in vitro produced P. berghei gametocytes and for estimating the number of ookinetes in relation to gametocyte numbers. These methods enabled us to study aspects of functional maturity of gametocytes (i.e. the capability to produce gametes and subsequent zygotes) independent of the mosquito.
In rodent malaria parasites the relation between functional maturity and morphology of gametocytes is poorly understood. In P. yoelii, differences in the morphology were observed between microgametocytes in blood ingested by mosquitoes and microgametocytes in blood taken from the tail of infected mice. It was suggested that the infective gametocytes are distributed preferentially within the capillaries of the vertebrate host (Landau, Miltgen, Boulard, Chabaud & Baccam, 1979). We used our technique for in vitro ookinete formation to investigate the spatial distribution of functionally mature gametocytes of P. berghei within the vertebrate host.

Materials and Methods

In vitro ookinete formation

For in vitro ookinete formation culture media RPMI 1640 (Gibco; 10.41 g/l) and MEM Earle's Salts (Gibco; 9.53 g/l) were used, without bicarbonate, both supplemented with inactivated foetal calf serum (10% v/v) and Hepes (5.94 g/l) and adjusted to pH 7.8 - 8.0 with 1 M NaOH. MEM has successfully been used for culturing ookinetes by other workers (Weiss & Vanderberg, 1977). RPMI 1640 was chosen since it is suitable for culturing erythrocytic stages of P. berghei (Janse, Mons, Croon & Van der Kaay, 1984). Infected erythrocytes, obtained as described below, were added to the media to give a suspension with a cell density of about 2×10^8 cells/ml. These suspensions were incubated at 20 ± 1°C and 80-90% humidity in 24-well culture plates (Costar, Cambridge, USA; 2 ml/well) or in 96-well microtitre plates (Nunc, Denmark; 0.2 ml/well).

Gametocytes from different blood sources

In vivo formed gametocytes were obtained from Swiss mice (20-25 g), 7 days after intraperitoneal infection with $0.8\text{-}1.2 \times 10^5$ P. berghei (ANKA-strain) infected erythrocytes. The ANKA-strain of P. berghei had been maintained by frequent transmission through Anopheles atroparvus (Van der Kaay & Boorsma, 1977). Only parasites derived from a second or third mechanical passage were used.

Heartblood (0.8 ml) was collected by cardiac puncture under ether anaesthesia and suspended in 5 ml of heparinized (2 i.u./ml) RPMI 1640 medium, pH 7.3 (Mons, Janse, Croon & Van der Kaay, 1983). This suspension was washed (200 g, 10 min) once with non-heparinized medium and the packed cells were resuspended in medium at pH 7.8-8.0.

Tailblood was collected in heparinized capillary tubes (Propper, Long Island City, N.Y.) and immediately suspended in medium at pH 7.8 - 8.0.

Blood ingested by mosquitoes: Anopheles atroparvus females (S-strain; Van der Kaay & Boorsma, 1977) were allowed to feed for a period of half an hour on a restrained mouse. Within 1 min. after feeding, fully engorged

mosquitoes were collected, midguts were dissected and the contents of the midguts of individual mosquitoes were mixed with medium at pH 7.8 - 8.0 and dispensed each into a separate well of a microtitre plate.

In vitro produced gametocytes were obtained from short-term in vitro cultures of the erythrocytic stages of P. berghei (ANKA-strain), established according to Janse, Mons, Croon & Van der Kaay (1984). Only once (20h after start of the culture) fresh erythrocytes were added to the cultures. After 65h the cultured cells were centrifuged at 200 g for 10 min to remove the medium. The packed cells were resuspended in RPMI 1640 at pH 7.8 - 8.0.

Parasite counts

After suspending infected erythrocytes in medium at pH 7.8-8.0 for 21-24h, samples with an erythrocyte density of approximately 8×10^7 cells/ml were taken from the wells and the number of ookinetes in 0.8-2.4 $\times 10^5$ erythrocytes was counted in a Burker haemocytometer. At a magnification of 400x mature ookinetes could easily be distinguished from other cells by their "banana"-shape and by the presence of pigment granules. In each well we established the ookinete yield. The ookinete yield is obtained by dividing the number of ookinetes/10^5 r.b.c. by the number of exflagellations/10^5 r.b.c. and multiplying this ratio by 100.

The number of exflagellations was counted before adding the infected erythrocytes to medium at pH 7.8-8.0. A sample of the infected erythrocytes was mixed with medium RPMI 1640 at pH 7.8-8.0. After 10 min at 21°C this suspension, with a cell density of 0.8-1.2 $\times 10^8$ cells/ml, was transferred to a Burker haemocytometer. After the cells had settled, the number of exflagellations/0.8-1.2 $\times 10^5$ erythrocytes was counted at a magnification of 400x.

Ookinete numbers are not presented in relation to the number of (macro)gametocytes but to the number of exflagellations since counting of (macro)gametocytes in smears not only is time-consuming but also is hampered by the non-random distribution of gametocytes. In Giemsa-stained

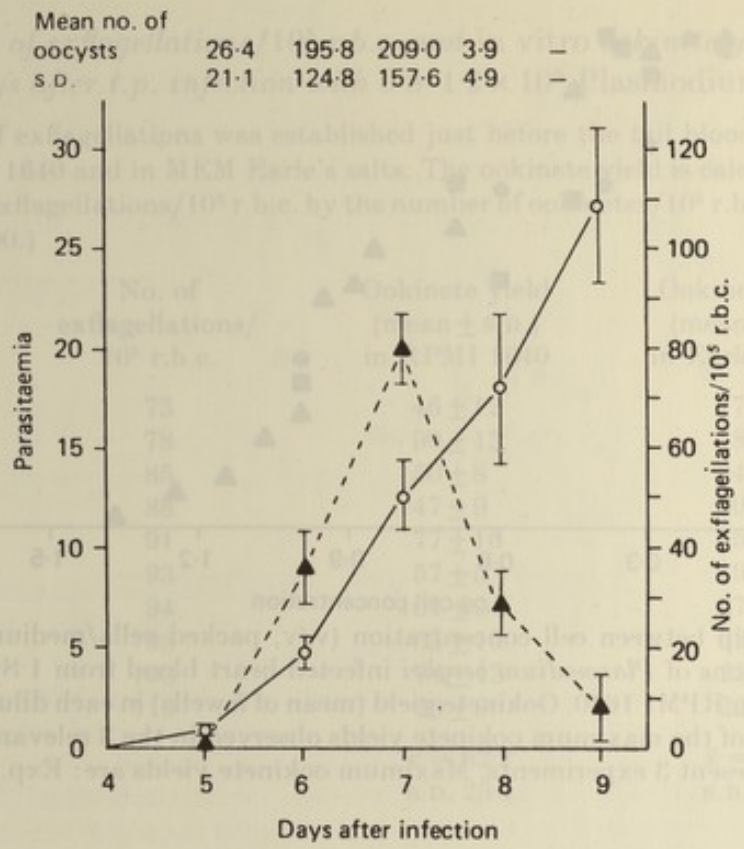

Fig. 1. *Plasmodium berghei* (ANKA-strain) infections in Swiss mice ($n = 10$). ○, Mean percentage of erythrocytes infected; ▲, mean number of exflagellations/10^5 r.b.c. Mice were infected intraperitoneally with 0·8–1·2 × 10^5 infected r.b.c. Oocyst numbers were established in *Anopheles atroparvus* mosquitoes 10 days after the infective bloodmeal. On each mouse 20–30 females, 7 days post-emergence, were fed on days 5–9 p.i.

smears of tail blood the percentage of infected erythrocytes was determined in 4-5 x 10^3 erythrocytes.

Results

In vivo produced gametocytes

For in vitro ookinete formation blood was collected on day 7 post-infection (p.i.) from Swiss mice showing a rapid increase of infected erythrocytes from day 4 to day 7 p.i. The characteristic course of parasitaemia and the number of exflagellations/10^5 r.b.c. in tail blood from 10 of these mice during the first 9 days p.i. are shown in Fig. 1.

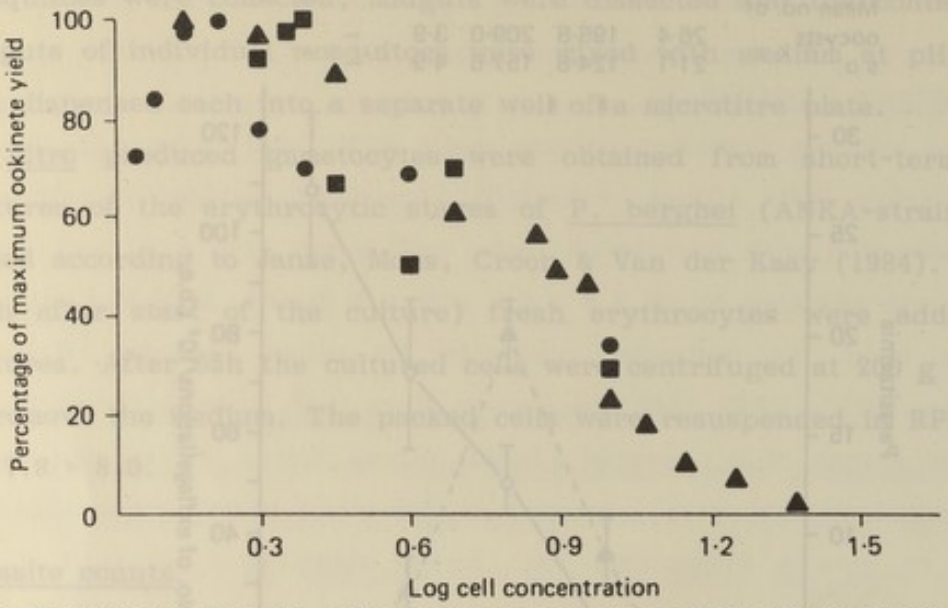

Fig. 2. Relationship between cell concentration (v/v, packed cells/medium) and ookinete yield. Serial dilutions of *Plasmodium berghei* infected heart blood from 1 Swiss mouse were made with medium RPMI 1640. Ookinete yield (mean of 5 wells) in each dilution is expressed as the percentage of the maximum ookinete yields observed in the 3 relevant dilution series. ●, ▲ and ■ represent 3 experiments. Maximum ookinete yields are: Exp. ●, 49; Exp. ▲, 50; Exp. ■, 39.

Maximum numbers of exflagellations in tail blood directly incubated in RPMI 1640 at pH 7.8-8.0 were reached on day 7 p.i. and ranged from 67 to $115/10^5$ r.b.c. On days 6 and 7 p.i. the number of exflagellations was established in two blood samples taken from the tail within a short interval (10 min). Between these samples a maximum difference of 10% was observed (mean of 20 observations 5.5%). In mosquitoes fed on days 5-9 p.i. maximal oocyst production was found on days 6 and 7 (Fig. 1), indicating the presence of functionally mature gametocytes.

Standardization of the method for in vitro ookinete formation

In three experiments we investigated the influence of erythrocyte concentration on ookinete yield. Serial dilutions of the packed cells of infected heart blood were made with RPMI 1640 at pH 7.8-8.0, resulting in cell concentrations (v/v packed cells/medium) as indicated in Fig. 2. Each dilution series was made with blood from a different mouse. In this case

Table 1. *Number of exflagellations/10^5 r.b.c. and* in vitro *ookinete yields in tail blood of Swiss mice 7 days after i.p. infection with* 0·8–1·2 × 10^5 Plasmodium berghei *parasites*

(The number of exflagellations was established just before the tail blood was incubated in medium RPMI 1640 and in MEM Earle's salts. The ookinete yield is calculated by dividing the number of exflagellations/10^5 r.b.c. by the number of ookinetes/10^5 r.b.c. and multiplying this ratio by 100.)

Exp.	No. of exflagellations/ 10^5 r.b.c.	Ookinete yield (mean ± S.D.) in RPMI 1640	Ookinete yield (mean ± S.D.) in Earle's MEM
1	73	46 ± 12	17 ± 4
2	78	90 ± 13	48 ± 9
3	85	50 ± 8	24 ± 6
4	88	47 ± 9	30 ± 9
5	91	77 ± 10	55 ± 6
6	93	57 ± 5	19 ± 5
7	94	104 ± 9	67 ± 9
8	98	53 ± 10	32 ± 7
9	103	74 ± 13	25 ± 8
10	119	26 ± 9	22 ± 9
		$\bar{x}$ = 62·2	$\bar{x}$ = 33·9
		S.D. 23·4	S.D. 16·9

ookinete yields are expressed as percentages of the maximum ookinete yield observed in the relevant dilution series. The results show that ookinete yields increased with higher dilutions to a cell concentration of 2%. Therefore we used a cell concentration equivalent to an r.b.c. density of about 2 x 10^8 cells/ml in the experiments described below.

In 10 experiments tail blood was incubated in both RPMI 1640 and MEM. MEM yielded 15-67% less ookinetes compared to RPMI 1640 (Table 1). Table 1 shows that the variation in ookinete yield between different wells containing blood from one mouse was small (s.d. 5-13 in RPMI 1640) compared to the differences in ookinete yield between blood collected from different mice (s.d. 23.4 in RPMI 1640).

To establish a possible relationship between number of gametocytes/10^5 r.b.c. and ookinete yield we altered the number of gametocytes/10^5 r.b.c. artificially by serially diluting infected heart blood from one mouse with a suspension of uninfected erythrocytes before the heart blood was incubated

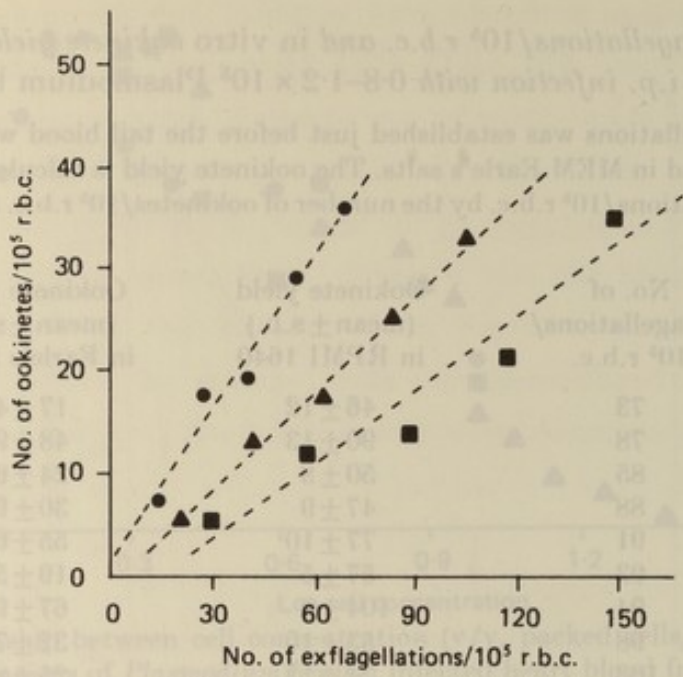

Fig. 3. Relationship between the number of exflagellations/10^5 r.b.c. and numbe. of *in vitro* produced ookinetes/10^5 r.b.c. In each experiment *Plasmodium berghei* infected he. t blood from 1 Swiss mouse was serially diluted with a suspension of uninfected r.b.c. before the blood was incubated in RPMI 1640. In all dilutions the final erythrocyte density was $1{\cdot}5–2 \times 10^8$ cells/ml. The ookinete number of each dilution is the mean of the ookinete number established in 5–10 wells. ●, ▲ and ■ represent 3 experiments. Exp. ●, $r = 0{\cdot}98$; $a = 0{\cdot}52$; $b = 0{\cdot}65$. Exp. ▲, $r = 0{\cdot}99$; $a = 0{\cdot}32$; $b = -1{\cdot}5$. Exp. ■, $r = 0{\cdot}96$; $a = 0{\cdot}96$; $b = -3{\cdot}5$.

in RPMI 1640. The resulting erythrocyte density was 1.5-2 x 10^8 cells/ml at all dilutions.

Results of three experiments (Fig. 3) show that ookinete yields remained constant irrespective of the gametocyte/erythrocyte ratio.

Morphology of developing ookinetes

Ookinetes could be distinguished from other cells in the haemocytometer by their banana-shaped appearance and clearly visible pigment granules 21-24h after start of the cultures. In 4 cultures we examined the morphology of developing parasites more thoroughly in Giemsa-stained smears since after culture for 21-24 h degenerated forms and young stages of ookinetes could be present but were unrecognizable in the haemocytometer. Smears were made every 3 h after suspending infected heart blood in RPMI

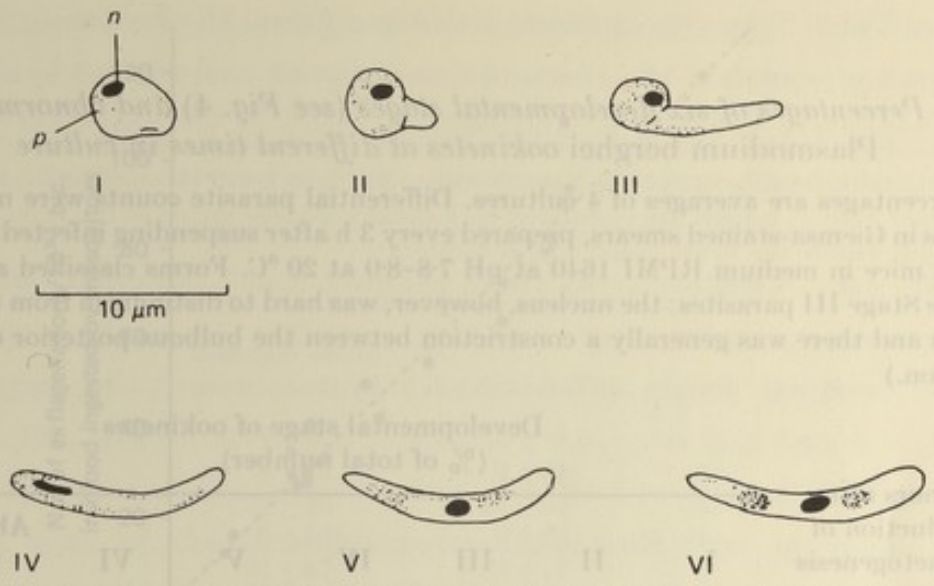

Fig. 4. A diagrammatic representation of the developmental stages of *in vitro* produced *Plasmodium berghei* ookinetes. The morphology was examined in Giemsa-stained smears at a magnification of 1000×. Stage I. Round or oval-shaped parasite. A more condensed appearance with a darker red staining and more extended nucleus (*n*) in comparison to macrogametes. Stage II. A little protrusion emerges from parasites resembling Stage I, filled with blue-staining cytoplasm without pigment granules. Stage III. First pigment granules (*p*) move into the enlarged protrusion. Nucleus still in the bulbous posterior end surrounded by pigment granules. Stage IV. First stage without round posterior end (banana-shaped). Nucleus migrates from the posterior end to the middle of the parasite. Stage V. This stage closely resembles Stage VI ookinete. Nucleus in the middle of the parasite. Dense pigment granules at one or both sides of the nucleus. Stage VI. Like Stage V but anterior end is enlarged and stains pink to red.

1640 and differential parasite counts were made on 200 parasites. Six different developmental stages of ookinetes (Fig. 4) were distinguished. Percentages of each stage are presented in Table 2.

We were not able to differentiate between unfertilized and fertilized macrogametes within 1h of activation of the macrogametocytes. After 3 h, however, parasites were seen with a more condensed appearance and with a darker red staining, more extended nucleus (Stage I). These parasites seemed to develop a protrusion (Stage II). After 15 h Stage I parasites had disappeared but parasites, presumably unfertilized macrogametes resembling the macrogametes seen during the first hour of culture, were still present. Stage III first appeared after 6 h. From 6 h onwards we constantly observed some parasites (maximally about 11%) resembling Stage III in which, however, the nucleus was hard to distinguish from the dark

Table 2. *Percentages of six developmental stages (see Fig. 4) and abnormal forms of* Plasmodium berghei *ookinetes at different times in culture*

(The percentages are averages of 4 cultures. Differential parasite counts were made on 200 parasites in Giemsa-stained smears, prepared every 3 h after suspending infected heart blood of Swiss mice in medium RPMI 1640 at pH 7·8–8·0 at 20 °C. Forms classified as abnormal resemble Stage III parasites: the nucleus, however, was hard to distinguish from the pigment granules and there was generally a constriction between the bulbous posterior end and the protrusion.)

Hours after induction of gametogenesis	Developmental stage of ookinetes (% of total number)						
	I	II	III	IV	V	VI	Abnormal forms
3	100·0	—	—	—	—	—	—
6	62·8	22·3	9·4	—	—	—	5·5
9	21·6	33·1	36·5	2·6	—	—	6·2
12	5·7	15·2	54·1	7·5	7·7	—	9·8
15	—	6·3	24·0	30·5	26·3	2·1	10·8
18	—	—	5·4	13·2	50·4	21·2	9·8
21	—	—	—	3·9	32·2	53·7	10·2
24	—	—	—	—	20·4	68·6	11·0

pigment granules. In addition, these parasites showed a constriction between the bulbous posterior end and the light-blue protrusion, and often the bulbous posterior end and the protrusion of these parasites were found separated in smears, indicating the fragility of these forms. We consider them to be abnormal or degenerated parasites. Stage VI is considered to be the mature ookinete since it persisted for at least 12 h in culture. In the four cultures 10-17% less ookinetes (all stages) were counted in smears at 24 h compared to 12 h, suggesting that some were lost during this period. From 21 h onwards only banana-shaped ookinetes and abnormal forms were seen.

Ookinete yields in different blood sources

Differential counts on 100-200 gametocytes in Giemsa-stained smears from tail blood of 10 mice indicated that 2-3 times more macro- than

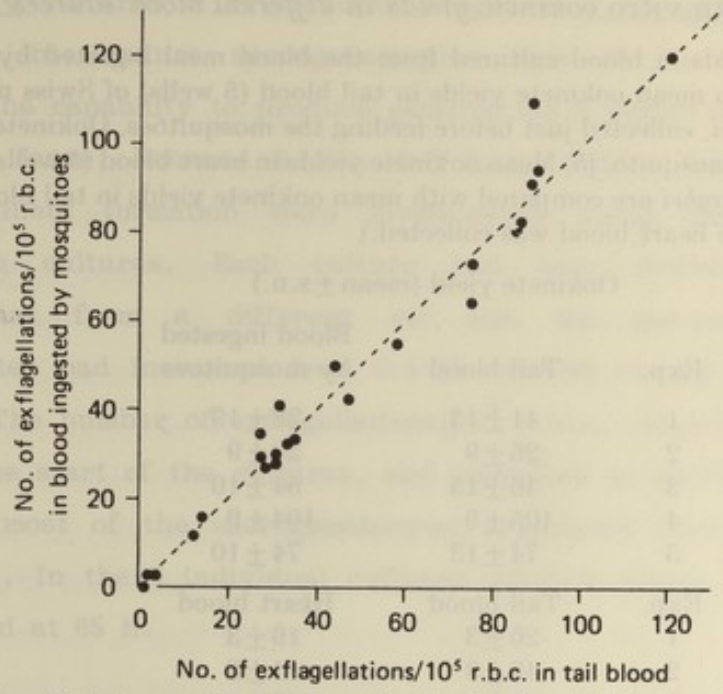

Fig. 5. Relationship between the number of exflagellations/10^5 r.b.c. in tail blood and in blood ingested by *Anopheles atroparvus* females. In each experiment the average number of exflagellations in blood ingested by mosquitoes ($n = 4$) is compared to the average number of exflagellations in tail blood of the *Plasmodium berghei* infected Swiss mouse on which the mosquitoes fed. Tail blood was collected just before and just after feeding the mosquitoes. The number of exflagellations was established/individual mosquito.

microgametocytes were present on days 6 and 7 p.i. (mean sex ratio ♀/♂ = 2.4). The percentage of erythrocytes infected with gametocytes on day 7 ranged between 0.2 and 0.5. Only gametocytes nearly or completely filling their host cell and sufficiently developed to allow sex differentiation were counted. Assuming that in the experiments shown in Table 1, too, 2.4 times more macrogametocytes than exflagellating microgametocytes were present, the percentage of female gametocytes transformed into ookinetes can be calculated from the number of exflagellations/10^5 r.b.c. at the time of withdrawal of the tail blood and the number of ookinetes/10^5 r.b.c. produced. This percentage ranged between 11 and 43% in RPMI 1640.

In experiments carried out on days 4-9 p.i., the average number of exflagellations/10^5 r.b.c. in blood ingested by mosquitoes (n=4) was compared to the average number in tail blood of the same mouse just before and just after feeding the mosquitoes (Fig. 5). In addition, mean ookinete

Table 3. In vitro *ookinete yields in different blood sources*

((*a*) Mean ookinete yields in blood cultured from the blood meal ingested by mosquitoes ($n = 8$) are compared to mean ookinete yields in tail blood (5 wells) of Swiss mice infected with *Plasmodium berghei*, collected just before feeding the mosquitoes. Ookinete yields were established/individual mosquito. (*b*) Mean ookinete yields in heart blood (5 wells) from Swiss mice infected with *P. berghei* are compared with mean ookinete yields in tail blood (5 wells), obtained just before the heart blood was collected.)

		Ookinete yield (mean ± S.D.)	
(*a*)	Exp.	Tail blood	Blood ingested by mosquitoes
	1	41 ± 13	38 ± 19
	2	26 ± 9	29 ± 9
	3	46 ± 15	54 ± 10
	4	105 ± 9	104 ± 9
	5	74 ± 13	74 ± 10
(*b*)	Exp.	Tail blood	Heart blood
	1	20 ± 3	19 ± 3
	2	46 ± 3	44 ± 8
	3	41 ± 1	49 ± 4
	4	21 ± 3	24 ± 6

yields in blood cultured from blood meals ingested by mosquitoes (n=8) feeding on mice 7 days p.i. were compared to ookinete yields in tail blood collected just before the mosquitoes were fed on these mice (Table 3a). No significant differences were found between blood ingested by mosquitoes and tail blood of the same mouse, either in number of exflagellating microgametocytes or in ookinete yield. Since the number of exflagellations in blood ingested by mosquitoes corresponded to the number in tail blood, the ookinete yields in both blood sources were calculated by dividing the number of ookinetes by the number of exflagellations established in tail blood just before the mosquitoes were fed. Assuming the same sex ratio as above (♀/♂=2.4), 11-44% of female gametocytes in tailblood and 12-43% of those in bloodmeals reached the mature ookinete stage in vitro.

In four experiments ookinete yields in heart and tail blood of the same mouse were compared. Heart blood was collected and treated in the same way as the tail blood, collected just before the heart puncture. The number of exflagellations differed maximally 16% between the two blood sources and only small differences were observed in ookinete yield (Table 3b).

In in vitro cultures of erythrocytic stages of P. berghei, development of ringforms into mature microgametocytes took 26 h; these gametocytes retained the capability to produce gametes for a period of approximately 26 h (Mons, Janse, Boorsma & Van der Kaay, 1984). Exflagellation and in vitro ookinete formation were investigated using gametocytes from 4 short-term cultures. Each culture had been initiated with infected erythrocytes from a different rat and the percentage of infected erythrocytes had increased from 0.5-1% to 6-8% during the first 24 h of culture. The number of exflagellations/10^5 r.b.c. ranged from 0 to 4 at 40 h after the start of the cultures, and increased to 22-78 at 65 h, indicating that most of the microgametocytes originated from in vitro invaded merozoites. In the 4 individual cultures ookinete yields of 4, 7, 16 an 22 were found at 65 h.

Discussion

Weiss & Vanderberg (1977), using essentially the same method for in vitro formation of ookinetes, estimated the percentage of macrogametocytes transformed into ookinetes at 1%, which is far less than the percentages estimated in this study (8-44%). They diluted infected blood from hamsters with a gametocytaemia of 3-10% six times with MEM. In our hands MEM yielded 20-60% less ookinetes than RPMI 1640. Moreover ookinete yields increased drastically with higher dilutions of the infected blood. Since gametogenesis will take place before the cells are settled in the wells, the release and mobility of microgametes may be less obstructed by other cells in the cultures with low cell concentrations. Within the mosquito midgut, however, fertilization and ookinete development occur in cell concentrations equal to or higher than that of blood. In other experiments we compared in vitro ookinete yields with those in vivo. We found 75-97% less ookinetes/10^5 r.b.c. within the blood meal of mosquitoes 18 h after feeding, than in tail blood taken simultaneously from the mice on which the mosquitoes fed and incubated in vitro for the same period (unpublished

results). This suggests that formation of ookinetes within the midgut is less efficient than in culture since mosquito blood meals, if taken and cultured in vitro, produced equal numbers of ookinetes to tail blood.

Apart from a possible lower chance of fertilization in vivo, the subsequent development of the ookinete within the midgut can be inhibited by mosquito factors. Gass (1977) and Gass & Yeates (1979) showed that P. gallinaceum ookinete development could be interrupted at the stage of a retort-shaped, immature form, by factors produced in the midgut of mosquitoes during blood digestion. In our cultures only small numbers of such retort-shaped forms (Stage III) failed to develop into banana-shaped stages. Gao & Yuan (1982) described the in vitro development of ookinetes of the related parasite P. yoelii. They observed the first mature ookinetes after culture for 7 h. Several young stages were described having a constriction between the protrusion and a bulbous posterior end. In our cultures these forms were absent or present in low numbers and were obviously fragile. Therefore we classified them as abnormal or degenerated. In several characteristics these forms resemble degenerated ookinetes of P. gallinaceum (Gass & Yeates, 1979).

In cultures with low erythrocyte concentrations a linear relationship existed between the number of gametocytes and the number of ookinetes when blood from an infected mouse was serially diluted with a suspension of uninfected erythrocytes. Thus the probability of fertilization did not decrease with decreasing gametocytaemia. Nevertheless considerable differences in ookinete yields were found between blood from different mice. This implies that exflagellation is not a reliable indicator of the capability of gametocytes to produce ookinetes.

In vitro ookinete formation excludes mosquito influences on the process of fertilization and ookinete production. Since our methods for in vitro ookinete formation and for estimating the ookinete yield gave reproducible results, these are useful tools in the study of functional maturity of gametocytes produced either in the vertebrate host or in in vitro cultures of the erythrocytic stages. We were able to obtain ookinetes from in vitro produced gametocytes, showing their functional maturity. Reproducible in

in vitro ookinete formation using cultured gametocytes has not been reported earlier, although feeding of cultured gametocytes of *P. falciparum* to mosquitoes resulted in oocyst production (Sinden, 1983).

The relation between functional maturity and the morphology of *P. berghei* gametocytes is not clear. Landau *et al.* (1979) compared the morphology of *P. yoelii* microgametocytes in blood taken from the tail of mice and from mosquito midguts and showed that, on morphological criteria, the gametocyte population within the mosquito blood meal differs from that in tail blood.

In *P. berghei*, closely related to *P. yoelii*, we on the contrary found no evidence for a non-random distribution of functionally mature gametocytes. Both the number of exflagellating microgametocytes and the *in vitro* ookinete yield in tail blood corresponded with those in heart blood and in blood ingested by mosquitoes.

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Chapter VI

DEVELOPMENT OF *PLASMODIUM BERGHEI* OOKINETES IN THE MIDGUT OF *ANOPHELES ATROPARVUS* MOSQUITOES AND *IN VITRO*

C.J. Janse, R.J. Rouwenhorst, P.F.J. van der Klooster, H.J.van der Kaay and J.P. Overdulve. 1985.
Parasitology, *91*, 219-225.

Summary

Plasmodium berghei ookinete formation *in vitro* and within the midgut of susceptible *Anopheles atroparvus* were compared. No significant morphological differences were seen, except that *in vitro* development was more synchronized and less degenerating forms occurred. *In vitro* ookinete yields were 4-31 times higher and less variable than those *in vivo*. Mosquitoes of a susceptible and of a refractory line of *A. atroparvus* were simultaneously fed on the same host or via a membrane with the same suspension of *in vitro*-formed mature ookinetes. Up to 100% of mosquitoes of the susceptible line produced oocysts, mostly in high numbers, whereas infection rates and numbers of oocysts produced in mosquitoes of the refractory line were lower and much more so after host feeding than after membrane feeding of mature ookinetes, indicating that refractoriness does not depend on a single process of inhibition.

Introduction

The number of Plasmodium oocysts produced in vector mosquitoes is only a small proportion of the total number of macrogametocytes ingested (Vanderberg, Weiss & Mack, 1977). We observed maximal oocyst production of P. berghei in susceptible Anopheles atroparvus mosquitoes, fed during the early days of infection, when infectivity of gametocytes is highest (Vanderberg & Gwadz, 1980) and calculated that an average of only 0.36% and at most 3.5% of the ingested macrogametocytes transformed into oocysts. When the same blood immediately after ingestion by mosquitoes was incubated in vitro, however, 12-43% of the mature macrogametocytes transformed into ookinetes (Janse, Mons, Rouwenhorst, Van der Klooster, Overdulve & Van der Kaay, 1985). Moreover, we found that functionally mature gametocytes (i.e. capable of producing gametes and subsequent ookinetes) are distributed at random within the vertebrate host. Hence, uneven spatial distribution of gametocytes within the rodent host or discrepancy between morphological and functional maturity of gametocytes (Landau, Miltgen, Boulard, Chabaud & Baccam, 1979) cannot explain the markedly low oocyst yield in susceptible mosquitoes.

Other factors that may be incriminated are (a) products in the blood of the vertebrate host, either of host or of parasitic origin (Eyles, 1951; 1952; Mendis & Targett, 1979; Carter, Gwadz & Green, 1979; Rosenberg & Koontz, 1984) or produced during blood digestion in the mosquito (Gass & Yeates, 1979) or physical condition within the midgut lumen, which may affect the viability of macro- and microgametes, the efficiency and rate of exflagellation, the fertilization process and/or the subsequent development of the resulting zygote and ookinete; and (b) factors interfering with the passage of the ookinete through the midgut wall and its early development into an oocyst.

The experiments described in the present paper were carried out (1) to evaluate the relative contribution of both groups of factors to reduced oocyst production in mosquitoes by comparing the morphology and the efficiency of ookinete formation in susceptible mosquitoes with those in

vitro; and (2) to compare oocyst production in susceptible and refractory A. atroparvus lines after infection by membrane feeding with in vitro developed mature ookinetes.

Materials and Methods

In vitro ookinete formation

In vivo-formed gametocytes were obtained from Swiss mice (20-25 g), 4-5 days after intraperitoneal (i.p.) infection with about 1 x 10^6 P. berghei (ANKA-strain) infected erythrocytes (r.b.c.). These mice had been injected i.p. with 100 µg/g body weight phenylhydrazine-HCl 4 days before to stimulate reticulocytosis. Tail blood and heart blood were collected and processed as described by Janse et al. (1985). The number of exflagellations/10^5 r.b.c. at 10 min, and of ookinetes/10^5 r.b.c. at 18 h after transfer of gametocytes to RPMI 1640 at pH 7.8-8.0, were counted; the ookinete yield is expressed as the number of ookinetes/100 exflagellations.

Comparison of in vivo and in vitro ookinete development

Susceptible A. atroparvus females (7-14 days post-emergence) were fed for 30 min on a Swiss mouse i.p. infected 7 days before with 1 x 10^5 parasites. Mosquitoes were fed and kept at 20±1°C. At 3 h intervals, up to 24 h after termination of the feeding, 3-5 fully engorged mosquitoes were dissected. The contents of each individual midgut were mixed with 10 µl of medium, spread on a microscope slide and stained with Giemsa. Differential parasite counts were made on 100-200 zygotes or ookinetes/slide.

Immediately after collecting tail blood for in vitro incubation, mosquitoes were fed on the same mouse for 30 min and then kept at 20±1°C. The number of ookinetes formed in the midguts of 10-12 fully engorged mosquitoes was estimated 16-18 h later i.e. after ookinetes were sufficiently differentiated to be recognized in the haemocytometer but before they may start disappearing by penetrating the midgut wall (Garnham, Bird, Baker,

Desser & El-Nahal, 1969; Speer, Rosales-Ronquillo & Silverman, 1975). The contents of each dissected midgut were thoroughly mixed with 200 µl of medium before transfer to the haemocytometer. Since the gametocyte composition of blood ingested by mosquitoes is the same as in tail blood (Janse et al., 1985), the yield of ookinetes developed within the midgut could be calculated with reference to the number of exflagellations/10^5 r.b.c. in tail blood. However, during the 16-18 h period of ookinete development the number of erythrocytes within midguts of fully engorged mosquitoes decreased from an average of 2.9 x 10^7 r.b.c. (range 2.2-3.6x10^7, n=40), 2 h after feeding on mice, to an average of 1.7 x 10^7 erythrocytes (range 1.2-2.6x10^7, n=40), 18 h after feeding. The slight weight loss of the mosquitoes during this period and the presence of abundant pigment granules at 18 h after feeding indicated that this erythrocyte reduction is not due to excretion but rather to blood digestion. As a consequence, the calculated in vivo ookinete yields should be considered over-estimated, on an average 1.7 (2.9/1.7) times and maximally 3 (3.6/1.2) times.

Feeding of in vitro-developed ookinetes to mosquitoes

Preliminary experiments showed the following procedure to be the most satisfactory. Heart blood, 1-2 ml, after incubation for 18 h in medium as described, was centrifuged (200 g, 10 min), resuspended in 20-40 ml of medium, layered on top of a 50% (v/v) Percoll/medium mixture and centrifuged for 10 min at 350 g. Erythrocytes settled to the bottom of the tube. The cells present at the interface, mainly ookinetes, unfertilized macrogametes and leucocytes, were collected, washed (200 g, 10 min) with medium, and mixed with 1-2 ml of heart blood from uninfected mice to get a 30% erythrocyte suspension. After counting the number of ookinetes/10^5 r.b.c. this suspension, under continuous stirring at 30°C, was fed for 1-2 h to mosquitoes (7-14 days post-emergence) in a 1 ml capacity membrane feeding apparatus. Fully engorged mosquitoes containing on average 2.9 x 10^7 r.b.c. were kept at 20±1°C and examined for oocysts after 8 days. Mosquitoes of two lines of A. atroparvus, widely differing in susceptibility to P. berghei (Van der Kaay & Boorsma, 1977), as checked by us in

Table 1. *Percentages of* 6 *developmental stages and abnormal forms of* Plasmodium berghei *ookinetes within the midgut of* Anopheles atroparvus *at different times after feeding*

(Percentages are the averages of 4 experiments. The morphology of stages and abnormal forms in Giemsa-stained smears is as described for *in vitro*-developed ookinetes (Janse *et al.* 1985) except that in *in vivo*-developed stages V and VI the nucleus is not always centrally located.)

Time after feeding (h)	Developmental stages of ookinetes (% of total number)						Abnormal forms
	I	II	III	IV	V	VI	
3	100	—	—	—	—	—	—
6	67	31	2	—	—	—	—
9	17	22	44	2	—	—	15
12	6	20	52	6	—	—	16
15	3	6	25	18	27	—	21
18	—	2	7	6	47	23	15
21	—	—	—	3	42	42	13
24	—	—	—	—	42	50	8

advance by simultaneous feeding on the same infected mouse, were fed in this way with the same ookinete suspension.

Results

Comparison of in vivo and in vitro ookinete development

Morphologically, ookinete development in vitro (Janse et al., 1985) closely resembled development within the midgut of A. atroparvus (Table 1). However, in vitro development seemed to be more synchronous, maximally about half as many abnormal forms were found as in vivo and in vivo the nuclei of stages V and VI were not always located in the middle of the parasites.

In 5 experiments (Table 2) ookinete yields in mosquito midguts were, on average, 14 times (minimally 4, maximally 31 times) lower and much more variable than those in vitro.

Table 2. *Mean ookinete yields* (6 *wells*) *in tail blood from* Plasmodium berghei-*infected Swiss mice, and in the midgut of* Anopheles atroparvus *mosquitoes* ($n = 10$–12) *fed immediately after collecting tail blood*

(Ookinetes were counted 16–18 h after incubation or after feeding the mosquitoes, respectively. For calculation of the ookinete yield see Materials and Methods section.)

Exp.	No. of exflagellations/10^5 r.b.c. in tail blood	Ookinete yield*			
		In vitro (mean ± S.D.)	*cv*	*In vivo* (mean ± S.D.)	*cv*
1	182	40 ± 4	10	1·3 ± 0·7	54
2	202	114 ± 11	10	6 ± 3	50
3	392	52 ± 10	19	11 ± 5	45
4	467	75 ± 5	7	19 ± 13	68
5	562	77 ± 9	12	6 ± 6	100

* *cv*, Coefficient of variation = S.D./mean × 100.

Oocyst production in susceptible and refractory mosquitoes after feeding of in vitro-developed ookinetes

Results are shown in Table 3. On average, 93% of the mosquitoes of the susceptible line (S) became infected after host feeding, compared to only 18% of the mosquitoes of the refractory line (R) and in the latter oocyst numbers/mosquito were always very low.

After membrane feeding the same tendency was seen, although less pronounced. In the S-line, percentages of positive mosquitoes were comparable to those after host feeding, but the mean numbers of oocysts/mosquito were lower; in mosquitoes of the R-line these figures were higher after membrane feeding, or no difference between membrane and host fed mosquitoes occurred. In a few mosquitoes of the R-line relatively large numbers of oocysts (up to 93) were produced after membrane feeding, which was not observed after host feeding.

Table 3. Plasmodium berghei *oocyst yields in* Anopheles atroparvus *after host feeding and after membrane feeding of* in vitro-*developed ookinetes*

(Two lines of *A. atroparvus* were used which differ in susceptibility to the parasite (S: susceptible; R: refractory). Host feeding: mosquitoes of the S- and R-lines were simultaneously fed on a Swiss mouse, 6 or 7 days after i.p. infection with 10^5 parasites. Membrane feeding: Mosquitoes of the S- and R-lines were simultaneously fed with mature ookinetes in a 30% r.b.c. suspension. Ookinetes were produced *in vitro* by incubation of infected tailblood in medium at pH 7·8–8·0 for 18 h.)

Mode of infection	No. of ookinetes ingested/mosquito	Mosquitoes with oocysts (*n*)	No. of oocysts/positive mosquito	
			Mean	Range
	S-line			
Host feeding (4 exps)	—	93% (56)	206	2–676
Membrane feeding				
Exp. 1	1×10^4	77% (30)	9	1–59
Exp. 2	1×10^4	92% (12)	5	1–220
Exp. 3	3×10^4	100% (23)	69	5–203
Exp. 4	3×10^4	100% (20)	121	2–235
	R-line			
Host feeding (4 exps)	—	18% (49)	3	1–5
Membrane feeding				
Exp. 1	1×10^4	38% (34)	3	1–12
Exp. 2	1×10^4	58% (12)	3	1–13
Exp. 3	3×10^4	32% (31)	28	1–93
Exp. 4	3×10^4	16% (18)	19	3–51

Discussion

Since non-random distribution or low functional maturity of gametocytes cannot explain the low oocyst productivity in mosquitoes (see Introduction section), other processes during development in mosquitoes must be responsible.

(1) Gamete formation within the midgut of mosquitoes

We did not investigate whether ingested microgametocytes exflagellated at the same rate within engorged mosquitoes as they are able to do in vitro, but have no evidence to the contrary. The same can be said for the

process of differentiation of macrogametocytes to macrogametes after engorgement.

(2) Fertilization

In dilution experiments in vitro (Janse et al., 1985) the ookinete yield obtained in the most concentrated cell suspension (3×10^9 cells/ml) was only 3% of the maximum yield, obtained at a 10 times lower cell concentration. We argued that increasing obstruction of the mobility of microgametes by erythrocytes may account for this drastic reduction in ookinete yield. If this is correct the chance of male-female contact within the midgut, in which the cell concentration is about 1×10^{10} cells/ml, must be very low, and probably is the main factor limiting the number of mature ookinetes in vivo.

(3) Ookinete development

During the 24 h period of ookinete development we never observed large numbers of morphologically abnormal stages (Table 1). It seems unlikely, therefore, that impairment of zygote development in vivo causes drastic losses, as was suggested for P. gallinaceum by Gass & Yeates (1979).

(4) Passage of the midgut wall and subsequent development

We calculated that in spite of the 4-31 times lower ookinete yield in vivo than in vitro, 8×10^2 to 5×10^4 (mean 11×10^3) ookinetes developed in susceptible mosquitoes in our experiments. Oocyst numbers in these mosquitoes ranged between 10 and 2000, indicating that only a small fraction of mature ookinetes developed into oocysts. The same is obvious from the results of membrane feeding of in vitro-produced mature ookinetes to susceptible mosquitoes. Ifediba, Weiss & Vanderberg (1982), who fed in vitro-formed P. berghei ookinetes to A. stephensi mosquitoes, also observed that the majority of ingested ookinetes failed to develop into oocysts.

In conclusion, there are at least two bottle-necks for macrogametes of P. berghei in mosquitoes before attaining the oocyst stage. One occurs during

development in the midgut lumen and is most probably mainly the result of a reduced chance of male-female contact and fertilization and, to a much lesser extent, of aberrant zygote development.
The second occurs during or after penetration of the midgut wall.
The experiments in which the same suspension of in vitro-developed ookinetes was fed to susceptible and refractory mosquitoes suggest that refractoriness of these mosquitoes is not primarily dependent on factors which reduce the rate of fertilization or viability of ookinetes during their development within the lumen of the midgut, but on later development, as is also suggested by histological data of J.F. Sluiters (personal communication).
Nevertheless, the differences between S- and R-mosquitoes is less pronounced after membrane feeding than after host feeding and in some of the mosquitoes of the R-line remarkably large numbers of oocysts were produced after membrane feeding of ookinetes; after host feeding this is very exceptional. These observations indicate that several factors, acting on different parts of the sexual and sporogonic cycle, inhibit parasite development in mosquitoes of the refractory line.

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Chapter VII

RAPID REPEATED DNA REPLICATION DURING MICROGAMETOGENESIS AND DNA SYNTHESIS IN YOUNG ZYGOTES OF PLASMODIUM BERGHEI

C.J. Janse, P.F.J. van der Klooster, H.J. van der Kaay, M. van der Ploeg and J.P. Overdulve. 1986.
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Introduction

Evidence has accumulated that in malaria parasites, as in other Eucoccidia, meiosis occurs during sporogony; except for the zygote all stages, from sporozoite to young gametocytes, are considered to be haploid. In contrast, and in spite of its reported Feulgen-negativity (Canning & Sinden, 1975; Sinden, 1978) - assumed to be caused by some DNA-"masking" nucleoprotein(s), but recently contradicted by Cornelissen et al. (1984a) - the mature microgametocyte in the blood of the vertebrate host has been considered octoploid. This was concluded from (i) the very short time (a few minutes) required after activation to produce the eight microgametes (Canning & Sinden, 1975), (ii) the lack of inhibition of exflagellation by mitomycin-C, used as a specific DNA synthesis inhibitor, at concentrations far above those inhibiting schizogony (Toyé et al., 1977), (iii) ultrastructural studies on gametocytogenesis (Sinden, 1982; Sinden & Smalley, 1979) and microgametogenesis (Sinden, 1981, 1983a, b).
This communication presents preliminary results of cytophotometry of Feulgen-stained micro- and macrogametocytes before and during gametogenesis and zygotes of Plasmodium berghei both in the presence and absence of mitomycin-C or aphidicolin, a strong inhibitor of DNA polymerase-α, demonstrating DNA synthesis as indicated in the title.

Materials and Methods

Gametocytes of P. berghei (ANKA-strain) were obtained from tail blood of phenylhydrazine-treated mice, four to five days after infection (p.i.). Gametogenesis and ookinete development were performed in microplate wells in RPMI 1640 (Flow) pH 8.0, supplemented with fetal calf serum (10% v/v) and Hepes (5.94 g/l), (activating medium: A.M.) at 20 ± 1°C, according to Janse et al. (1985). Pre-incubation was done in RPMI 1640 pH 7.3 at 37°C in culture vessels, according to Janse et al. (1984). From 11 to 15 min after activation (p.a.) the number of exflagellating microgametocytes/10^5

erythrocytes was counted in a haemocytometer. DNA per cell was measured by direct fluorometry of Feulgen-stained cells using the same procedures and equipment as Cornelissen et al. (1984b). Intra-erythrocytic ring stages, less than three hours old, obtained from synchronized infections (Mons et al., 1985) were used as reference. In all cases slides, made of a suspension of such ring stages in PBS, were fixed, hydrolysed, stained and measured together with the slide of stages to be investigated. The frequency distribution of fluorescence values of these ring stages always showed a narrow, almost symmetrical Gauss distribution with a S.E.M. of about 0.5%. The mean of 30 to 50 ring stages was arbitrarily set at 100 fluorescence units (F.U.) and taken to represent the haploid value, as indicated by previous comparative measurements (not shown). Aphidicolin was obtained as a 60 mM solution in DMSO; controls of aphidicolin experiments, always contained 0.75% DMSO (no effect of this DMSO concentration alone was observed), equal to that in the highest aphidicolin concentration used. Micro- and macrogametocytes, before and after activation, could easily be distinguished from each other and from other parasite stages after Feulgen staining, by size and shape of cells (cell membranes remained faintly visible) and nuclei, position of nuclei within the cells, and size and distribution of pigment granules (details will be published elsewhere).

Results

DNA synthesis during gametogenesis.

In A.M. without inhibitors, fluorescence of microgametocytes increased within 10 min of activation from 177 F.U., before activation, to above the octoploid value (800 F.U.)(exflagellation started at about 11 min. p.a.), whereas macrogametocytes/macrogametes remained constant at about 150 F.U. during this period (Table I). The presence of mitomycin-C (Hoechst), 10 μg/ml, in the A.M., with or without additional pre-incubation of the cells with the drug (same concentration) for two to

Table I—Total DNA amount per cell, measured by direct fluorometry of Feulgen-stained cells, in mature microgametocytes (DNA synthesis) and macrogametocytes (no DNA synthesis) of *Plasmodium berghei* after activation (p.a.) in RPMI 1640 pH 8·0 at 20 ± 1°C without addition of DNA synthesis inhibitors. Mean fluorescence of young intra-erythrocytic ring stages was taken to represent the haploid complement and was arbitrarily set at 100 F.U. (for details see text)

Time p.a. (min)	DNA content: F.U. ± S.E.M (n)	
	microgametocytes	macrogametocytes
0	177 ± 3·1 (42)	141 ± 3·0 (32)
2	316 ± 9·1 (29)	—
4	484 ± 16·8 (43)	158 ± 5·6 (30)
6	602 ± 18·4 (30)	—
8	817 ± 20·4 (47)	149 ± 5·1 (40)
10	902 ± 12·1 (33)	146 ± 3·0 (33)
11	exflagellation starts	

six hours, had no effect at all on exflagellation or on the fluorescence values p.a. in either of the gametocytes, although a 10-fold lower dose of mitomycin in RPMI 1640 pH 7.3 at 37°C for 20 hours reduced schizogony for 50 to 70%, and most schizonts that did develop were morphologically degenerated.

However, when aphidicolin was added to the A.M., the number of exflagellations at 13 min p.a. dropped drastically with increasing drug concentrations and was only from 38% (10 μM aphidicolin) to 8% (450 μM) of that seen in DMSO-containing controls (on average 1346 exflagellations/10^5 erythrocytes) (mean of triple estimates).

Table II—Inhibition by aphidicolin of DNA synthesis in activated microgametocytes of *P. berghei*. Total DNA per cell was measured by direct fluorometry as in Table I at 10 min p.a.

Aphidicol concentration (μm)	DNA content: F.U. ± S.E.M. (n)
0	880 ± 6·6 (30)
10	601 ± 13·1 (30)
90	306 ± 5·1 (40)
450	200 ± 3·5 (40)

Equally, the increase of fluorescence in microgametocytes, measured at 10 min p.a., declined with increasing concentration of the drug (Table II). Instead of the whole nucleus, as in the controls, only a small area of the enlarged centrally located nucleus showed fluorescence in the presence of

Table III—Fertilization of macrogametocytes and DNA synthesis in zygotes, after transfer (p.a.) of mature gametocytes of *P. berghei* to activating medium without addition of DNA synthesis inhibitors. Total DNA per cell was measured by direct fluorometry as in Table I. Until 60 min p.a. cells were randomly sampled, hence n-ratios indicated relative prevalence of each of the three stages; at 3 and 24 hours p.a. only developing retort-shaped zygotes and mature ookinetes, respectively, were measured

	DNA content: F.U. ± S.E.M. (n)		
		Zygotes	
Time p.a.	Macrogametocytes	With two nuclei	With one nucleus
10 min	146 ± 3·0 (33)	(0)	(0)
20 min	148 ± 2·2 (37)	253 (2)	202 (1)
25 min	134 ± 2·1 (45)	230 ± 3·7 (20)	224 ± 4·6 (15)
60 min	126 ± 3·2 (9)	237 ± 4·1 (7)	237 ± 3·8 (29)
3 hours	—	(0)	381 ± 6·2 (53)
24 hours	—	(0)	368 ± 2·8 (25)

aphidicolin, but otherwise morphological development of microgametocytes (apart from reduced exflagellation) appeared normal. When aphidicolin, after 10 min of incubation, was removed from the A.M. by washing, exflagellation rose again considerably within 6 min of further activation. Also, pre-incubation of gametocytes with aphidicolin had no effect on microgametogenesis when the drug was removed before the cells were transferred to normal A.M. Inselburg & Banyal (1984) recently showed that aphidicolin has a similar reversible effect on DNA synthesis in asexual stages of P. falciparum.

Table IV— Inhibition by aphidicolin of DNA synthesis in zygotes of *P. berghei*. Aphidicolin was added to the medium one hour p.a. and DNA was measured as in Table I at 24 hours p.a.

Aphidicolin concentration (μM)	DNA content of zygote: F.U. ± S.E.M. (n)
0	368 ± 2·8 (25)
0·5	363 ± 5·7 (17)
1·5	365 ± 6·3 (10)
5	335 ± 4·1 (17)
15	262 ± 4·5 (13)
50	196 ± 6·9 (24)
150	206 ± 9·7 (15)

DNA synthesis in zygotes.

In controls without drug (Table III) nearly 50% of all macrogametes had higher than diploid fluorescence values (i.e. were fertilized) at 25 min

p.a., the greater part of them still with two distinct (male and female) nuclei.

About 30 min later, 80% of all macrogametes were diploid (zygotes), most of them already with a single nucleus. Again two hours later nearly all zygotes, now morphologically recognizable as developing ookinetes (Janse et al., 1985), had a mean fluorescence value of about the tetraploid value. They remained so for at least the next 21 hours (the maximum period investigated) during which time they developed to morphologically mature ookinetes. Addition of mitomycin (10 μg/ml) to the A.M. had no effect at all on ookinete development or on fluorescence values of the zygotes measured at 24 hours p.a., even when gametocytes were in addition pre-incubated with mitomycin for two to six hours. However, when aphidicolin was added to the A.M. one hour p.a., i.e. after most macrogametes had been fertilized (see Table III), fluorescence values at 24 hours p.a. dropped steadily with higher aphidicolin concentrations and at concentrations of 50 μM or higher the ookinetes, although morphologically mature, were still diploid, instead of tetraploid (Table IV).

Discussion and Conclusions

The results demonstrate:

(1) Mature microgametocytes of P. berghei are not octoploid before activation; their DNA content per cell lies between the haploid and the diploid value, as in macrogametocytes. Whether the DNA in excess of the haploid value reflects an onset of normal genome duplication or selective gene amplification - as has been assumed to occur in mature macrogametes of the related coccidian parasite Isospora (Toxoplasma) gondii (Cornelissen et al., 1984b) - remains to be determined.

(2) During gametogenesis microgametocytes of Plasmodium do synthesize DNA, up to about the octoploid value, steadily and at a very fast rate, only comparable to that known to occur in cleavage nuclei of higher eukaryotes.

(3) The time of fertilization of P. berghei in vitro could be pinpointed at about 15 min after exflagellation.

(4) Within two to three hours, fertilization is followed by DNA synthesis to about the tetraploid value, after which synthesis stops for at least 21 hours, although the zygote during this period develops to a morphologically mature ookinete. Sinden et al. (1985) found ultrastructural evidence of meiosis (synaptonemal complexes) in P. berghei within 2.5 hours of fertilization. If meiosis in Plasmodium is essentially comparable to that in higher eukaryotes, i.e., a two-step meiosis in which the formation of synaptonemal complexes is preceded by genome duplication, the above almost doubling of DNA during early zygote development must represent this pre-meiotic genome replication. Because DNA-values so far found in mature zygotes (368 F.U.) were slightly but significantly below the tetraploid value (400 F.U.) no definite conclusion can yet be drawn; alternatively, although we consider this less probable, the early DNA synthesis in zygotes might not represent total genome duplication but extended selective gene amplification, e.g. of genes coding for rRNA or for the huge amount of histones that will be necessary in subsequent sporogony.

At the light microscope level there is no evidence of nuclear division during ookinete maturation. However, in view of the spindle structures that have been found in ookinetes of P. gallinaceum (Mehlhorn et al., 1980) and P. berghei (Sinden et al., 1985) it is conceivable that intranuclear genome segregation occurs. This probably could be verified by scanning cytophotometry.

(5) The failure of mitomycin concentrations, inhibitory to asexual multiplication, to interfere with microgametocyte and ookinete development after activation is no proof of absence of DNA synthesis in these stages. Sinden et al. (1984) used a similar large difference in sensitivity to mitomycin (10 μg/ml) between asexual blood stages and (older) gametocytes to purify mature gametocytes of P. falciparum. This failure could be due to insufficient permeability of the cell membrane of gametocytes and zygotes for mitomycin. However, very high doses of mitomycin did have an instant

effect on exflagellation of microgametocytes (Sinden, personal communication).

Most probably, therefore, the failure should be explained by the mode of action of mitomycin-C: although it can inhibit DNA synthesis in eukaryotes (as well as in prokaryotes) very effectively by cross-linking both strands of DNA covalently (and hence also inhibiting transcription, which might be the main reason for its observed degenerative effect on asexual stages), it may only act in the cell as a cross-linking bifunctional alkylating agent after NADPH-dependent reduction of it quinone moiety (Waring, 1981). It might be, therefore, that mature microgametocytes and young zygotes of Plasmodium, in contrast to asexual stages and young gametocytes (Sinden et al., 1984), lack either the required quinone reductase (Szybalski & Iyer, 1967) or all NADPH-generating enzyme activity (pentose phosphate pathway, malic enzyme).

Aphidicolin, on the other hand, directly and specifically inactivates DNA polymerase-α (Kwant & Van der Vliet, 1980) - the enzyme responsible for chain elongation of eukaryotic nuclear DNA - and, as far as is known, does not interfere with, and is not dependent on other enzyme systems, proteins or other macromolecules.

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Chapter VIII

DNA SYNTHESIS IN PLASMODIUM BERGHEI DURING ASEXUAL AND SEXUAL DEVELOPMENT

C.J. Janse, P.F.J. van der Kloester, H.J. van der Kaay, M. van der Ploeg and J.P. Overdulve, 1986

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Summary

DNA contents of individual stages of Plasmodium berghei were measured by direct microfluorometry after Feulgen-pararosaniline (SO_2) staining. Sporozoites, intra-erythrocytic ringforms and trophozoites (until at least 15 h after invasion) are haploid and non-synthesizing DNA. DNA is synthesized just before and during schizogony, which takes 4-8 h. Genome duplication and segregation are alternating events throughout this process. Mature micro- and macrogametocytes have DNA contents between the haploid and diploid value; most, if not all of the DNA in excess of the haploid value is synthesized during the last 5-10 h of maturation. During gametogenesis microgametocytes within 8-10 min synthesize DNA rapidly and at a very high rate to more than the octoploid value while the DNA content of macrogametocytes remains constant. Fertilization in vitro takes place within 1 h after gamete formation. Within 2 h and coinciding with the onset of meiosis the zygote then synthesizes DNA up to almost the tetraploid value, after which synthesis stops during ookinete development. All the above mentioned processes of DNA synthesis are reversibly inhibited by aphidicolin (IC_{50} from 2-13 μM). From the rate of DNA synthesis

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during microgametogenesis we calculated a minimum of 1300 origins of replication in the haploid genome of P. berghei.

Introduction

Malaria parasites of mammals multiply by asexual schizogony in liver cells as well as in erythrocytes in which gametocytes (sexual cells) are also produced. After ingestion of these gametocytes by a vector mosquito, gamete formation (gametogenesis) is followed by fertilization of the macrogamete by one of the 8 microgametes emerging from the microgametocyte during exflagellation. The zygote transforms into a motile ookinete that encysts (oocyst) to produce the infective sporozoites.

DNA synthesis in intra-erythrocytic asexual stages of Plasmodium species has been investigated by incorporation of radiolabeled precursors [1-8]. In synchronized infections it could be demonstrated that in the 24h cycle of P. knowlesi [3] and P. chabaudi [5] as well as in the 48 h cycle of P. falciparum [6-8] the S-phase occurs during the later part of the asexual cycle.

DNA synthesis during sexual reproduction has never been demonstrated directly, but electron microscopic studies and experiments in which mitomycin-C was used as a specific DNA replication inhibitor [9,10] strongly suggested that both gametocytes replicate their genome during early development. Mature macrogametocytes, nevertheless, are assumed to be haploid, as a result of supposedly so-called cryptomitosis following replication, and mature microgametocytes are considered octoploid [9, 11, 12] in spite of the fact that both mature gametocytes have been reported to be Feulgen-negative [12, 13]. The latter observation, however, has recently been demonstrated to be a mere artefact due to sub-optimal reaction conditions [14].

Microgametogenesis itself, which occurs within a few minutes after ingestion of microgametocytes by a mosquito, is thought to be restricted to mitotic segregation of the 8 preformed haploid microgamete complements

[10, 13]. In zygotes the recent demonstration of synaptonemal complexes has suggested meiotic DNA replication during early ookinete development [15].

In contrast to biochemical investigations, cytophotometric methods allow measurement of the DNA content of individual cells that can be identified morphologically.

In this way DNA synthesis throughout the life cycle of the related coccidian Isospora (Toxoplasma) gondii has been analyzed [16]. With the same methods we have now been able to determine the exact DNA contents of successive stages of P. berghei and the time and rate of DNA synthesis during intra-erythrocytic schizogony and gametocyte development, and during gametogenesis and subsequent development of the zygote. Some of the latter results have already been published in a preliminary communication [17]. This paper gives a full account of our cytophotometric investigations on DNA synthesis in P. berghei. The results generally confirm previous conclusions with respect to DNA synthesis during schizogony, but require a revision of existing concepts about sexual reproduction of malaria parasites.

Materials and Methods

Parasites. The ANKA-strain of P. berghei, maintained by blood passage through rodents and frequent transmission through Anopheles atroparvus [18] was used.

(1) To investigate DNA synthesis during intra-erythrocytic development synchronized infections were established in Wistar rats by intravenous inoculations of in vitro cultured purified mature schizonts [19]. Parasitaemia in these rats increased rapidly and stabilized 3 h post inoculation (p.i.) at 1-3%, only ringforms being present. During the next 15 h all parasites developed into large trophozoites. About 19 h p.i. the first schizonts (with 2 nuclei) appeared. The numbers of schizonts and of nuclei per schizont then increased for about 3 h. At 24 h p.i. all schizonts had

disappeared from the circulation; only ringforms and young gametocytes were present. The first mature microgametocytes, capable of exflagellation, appeared at 26 h p.i. Their number increased within 4 h and remained stable for the following 20 h. From 26 h p.i. onward microgametocytes could be distinguished from macrogametocytes by a larger, more elongate nucleus and larger pigment granules. For a detailed description of parasite development in synchronous infections see ref. 19. At different periods p.i. tailblood from the rats was collected in heparinized capillary tubes and washed with phosphate-buffered saline (PBS) at pH 7.3 (200 x g, 5 min) to make thin blood films. Because developing schizonts disappear from the circulation [19], tailblood was collected at 18 h p.i. and incubated at 37°C in RPMI 1640 (Gibco) at pH 7.3 [20, 21] for 3 or 6 h, in 24 well plates placed on a shaker, after which thin blood films were made as above. Similar in vitro cultures were started at 3 h p.i., with or without addition of aphidicolin (see below). At 15 and 21 h after incubation (18 and 24 h p.i.) thin bloodfilms were made as above. In all cultures parasitaemia was stable during the 3 to 21 h period of cultivation.

(2) To investigate DNA synthesis during gametogenesis and zygote/ookinete development mature gametocytes were incubated in microplate wells at 20°C in RPMI 1640 at pH 8.0 for 24 h [18]. The gametocytes were obtained from tailblood of Swiss mice (20-25 g) treated with phenylhydrazine-HCl (100 mg (kg bodyweight)$^{-1}$) 4 days prior to i.p. injection with about 1 x 10^6 infected red blood cells (r.b.c.). In these infections parasite development is rather synchronous and gametocytaemia relatively high: on day 4 or 5 p.i. the numbers of morphologically mature macro- and microgametocytes (macro/micro-ratio varying from 1 to 2) increase about 4-10 fold within 4 to 6 h, and then remain stable for at least 15 h. Numbers of capacitated microgametocytes, estimated by in vitro exflagellation [18], roughly correspond with the numbers of morphologically mature microgametocytes. Ookinete yields (the number of mature ookinetes per 100 exflagellating microgametocytes [18]) also remain stable during the 15 h period and 20% or more of the macrogametocytes are functionally mature (data not shown in detail; a typical gametocytaemia on day 4 to 5

p.i. is shown in Fig. 1). Tailblood for all experiments was collected 4-6 h after the number of gametocytes had reached the plateau value, thus assuring that a large proportion of the gametocytes was functionally mature. During the first 10 min of culture slides were made without washing and always dried within 1 min; other slides were made as described above.

(3) Sporozoites were obtained from salivary glands of A. atroparvus dissected 20 days after the infective meal.

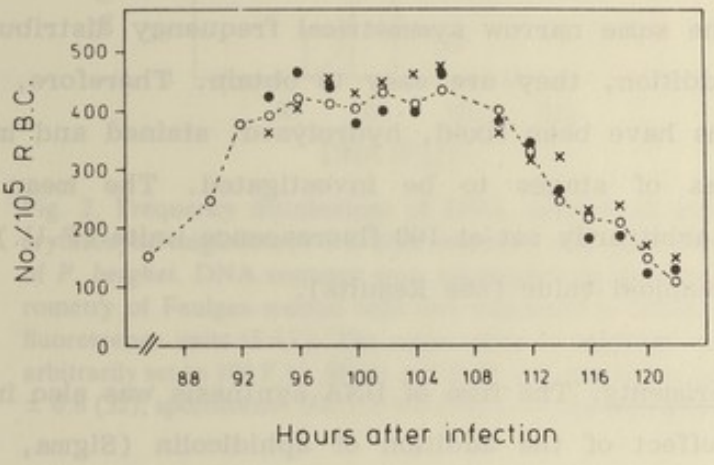

Fig. 1. A typical course of the number of gametocytes on day 4 and 5 p.i. in tailblood of a mouse, inoculated i.p. with ± 1 × 10^6 infected r.b.c., 4 days after phenylhydrazine-HCl treatment.
(○) Number of exflagellations counted in a haemocytometer, 13 min after incubation of tailblood in microwell plates at 20°C in RPMI 1640 at pH 8.0.
(x, •) Number of micro- and macrogametocytes, respectively, counted in Giemsa-stained bloodfilms. During the period of 108 – 120 h p.i. many degenerated forms [19] were observed which disappear rapidly from the circulation. Mean in vitro ookinete yields (= the mean number of ookinetes per 100 exflagellations [18], estimated in 3 wells) in tailblood, collected at 96, 100 and 104 h p.i. were 54, 56 and 52, respectively.

Cytophotometry. All blood films were fixed, hydrolysed and stained with Feulgen-pararosaniline (SO_2) as described earlier [14]. The DNA content of individual parasite nuclei was measured by direct microfluorometry using the same equipment and procedures as Cornelissen et al. [16], except that a 100x/1.30 NPL oil immersion objective (Leitz) was used. By adequate preadjustment, photodecomposition by pre-exposure of the cells to U.V.

light could be avoided completely. Standard exposure time was 1 s. The different parasite stages were easily recognized after Feulgen staining by size and shape of cells (cell membranes remained faintly visible) and nuclei, position of nuclei within the cells and size and distribution of pigment granules. In view of the very low DNA content of P. berghei [16] a reliable DNA-standard, i.e. constant and comparable in amount to the DNA to be measured, is of paramount importance. The nuclei of ringforms of P. berghei themselves showed to be the most useful standard: they always show the same narrow symmetrical frequency distribution (Results, Fig. 2); in addition, they are easy to obtain. Therefore, in all experiments ringforms have been fixed, hydrolysed, stained and measured along with the slides of stages to be investigated. The mean of 30 to 50 ringforms was arbitrarily set at 100 fluorescence units (F.U.) and taken to represent the haploid value (see Results).

Inhibition experiments. The time of DNA synthesis was also investigated by studying the effect of the addition of aphidicolin (Sigma, St. Louis), a very specific inhibitor of DNA polymerase-α [22-25], to cultures. Since the drug is dissolved in dimethylsulfoxide (DMSO), control cultures always contained the maximal DMSO concentration used (0.75%, v/v), but no effect of DMSO alone was observed, compared to cultures without any addition. The effect of mitomycin-C (Hoechst) on asexual and sexual development of P. berghei is to be published elsewhere in this journal [26].

Results

Sporozoites and ringforms (Fig. 2). Both stages had symmetrical distributions and exactly the same mean fluorescence value, lower than that of any other stage investigated except microgametes, which have the same mean value (see gametogenesis). This value, therefore, can be considered to represent the haploid complement (see Discussion).

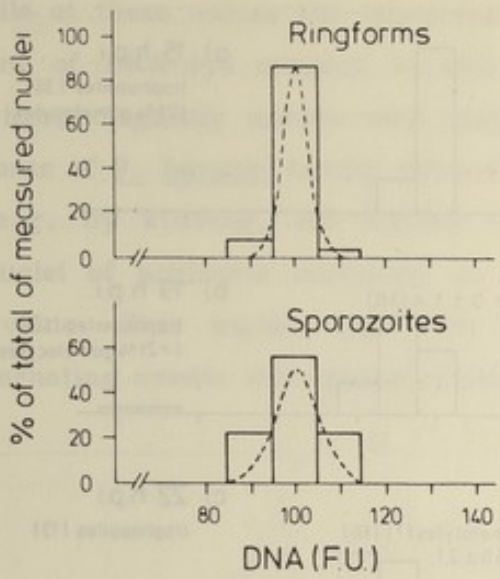

Fig. 2. Frequency distributions of DNA contents of intra-erythrocytic ringforms (0–3 h after invasion) and sporozoites of *P. berghei*. DNA contents were established by microfluorometry of Feulgen-stained cells and expressed in arbitrary fluorescence units (F.U.). The mean value of ringforms was arbitrarily set to 100 F.U. Mean ± S.E.M. (*n*): ringforms: 100 ± 0.8 (32); sporozoites: 100.5 ± 0.9 (30); (-----) normal curve.

Trophozoites and schizonts. Until 15 h p.i no DNA synthesis occurred in developing trophozoites (Table 1). From 19 h p.i. onward uninucleated trophozoites with up to the diploid value were observed (Fig. 3b, c), as well as a very few binucleated schizonts. At 22 h p.i., in vivo as well as in cultures started at 18 h p.i., most parasites were schizonts. Only a small fraction of the parasites was still uninucleated; an increasing part of the latter, therefore, must consist of young gametocytes (Fig. 3c, left).

TABLE I

Relative amounts of DNA cell^{-1} in randomly chosen ringforms and trophozoites of *P. berghei*

Hours post infection	Stage	DNA content (F.U.) mean ± S.E.M. (*n*)
2	ringform	100 ± 0.5 (40)
5	ringform/small trophozoite	103 ± 0.4 (35)
10	intermediate trophozoite	102 ± 0.6 (55)
15	large trophozoite	103 ± 0.7 (36)

[a] Parasites obtained from a synchronous infection in a rat at different hours after intravenous inoculation of cultured mature schizonts [19]. DNA contents were established as in Fig. 2. Counting of the number of gametocytes at 35 h p.i. [19] showed, retrospectively, that in this rat 21% of the parasites, measured until 15 h p.i., developed into gametocytes, although they are not distinguishable as such, morphologically, before 26 h p.i. (see also Fig. 3).

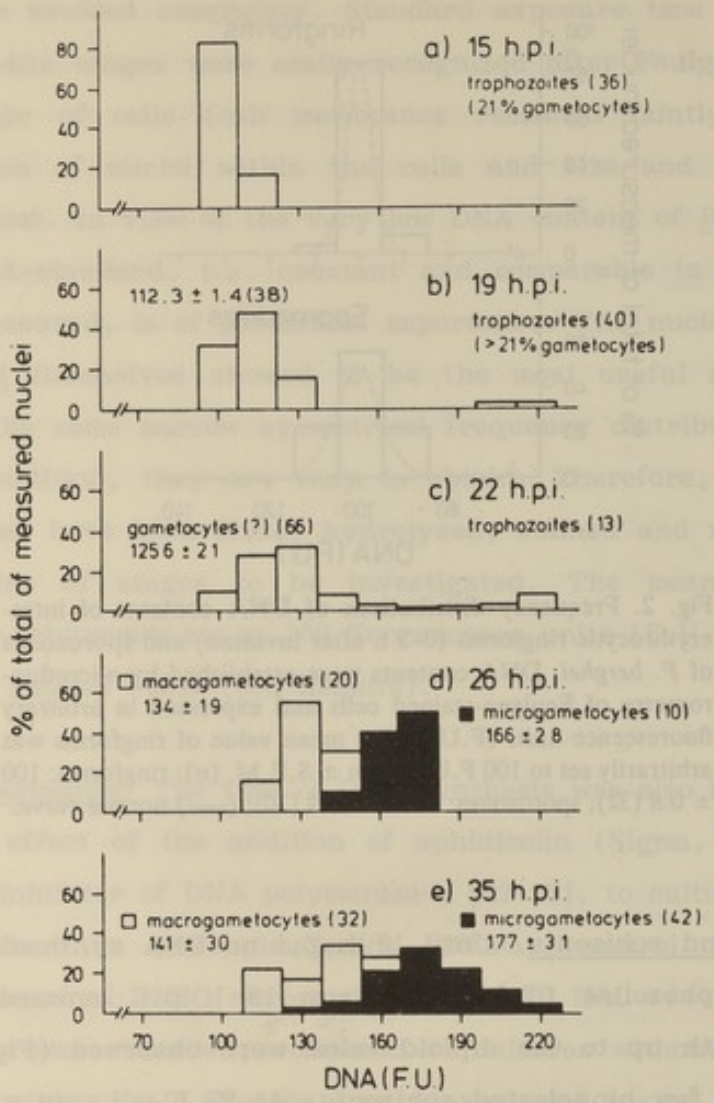

Fig. 3. Frequency distribution of DNA contents of randomly chosen *P. berghei* trophozoites (uninucleated) and gametocytes obtained at different hours p.i. from the same synchronous infection as in Table I. At 35 h p.i. 90% of the microgametocytes and 75% of the macrogametocytes were capable of exflagellation and of transforming into ookinetes, respectively.

Schizogony itself was studied in in vitro cultures started at 18 h p.i. Fig. 4 shows the total DNA content per cell in these schizonts in relation to the number of their nuclei. The amount of DNA per cell increased in proportion to the number of nuclei. Values higher than 20 times the haploid value, however, were never observed in schizonts with considerably more

than 10 nuclei, while at these values the relationship between fluorescence emission and amount of DNA-dye complex is still linear. Schizonts with 16-20 nuclei were morphologically mature with distinct merozoites (in culture, mature schizonts of P. berghei hardly release their merozoites unless shearing forces, e.g. by stirring, are applied [19]). In all stages of development the nuclei of schizonts contained on average not more DNA than the diploid value. This implies that DNA replication and genome segregation are alternating events throughout schizogony.

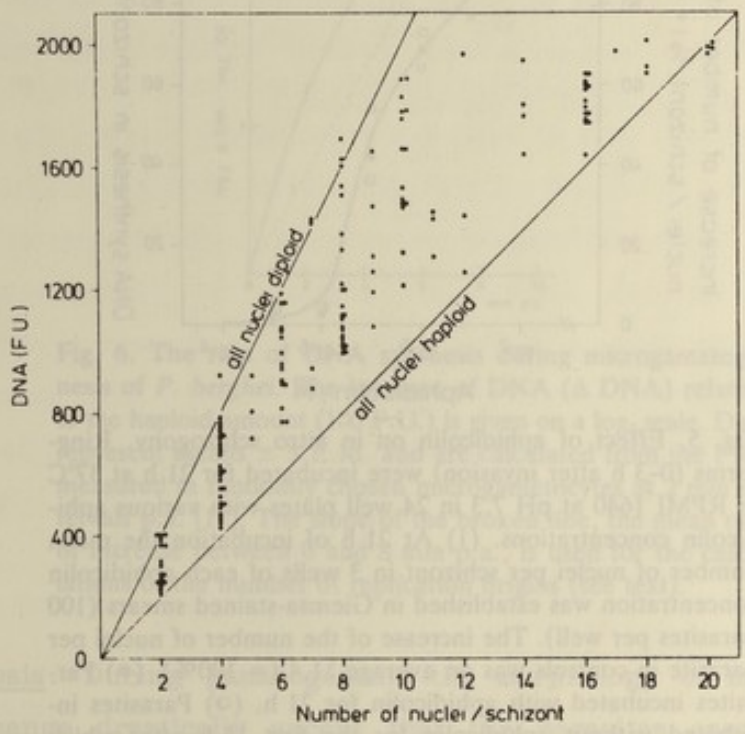

Fig. 4. DNA contents in randomly chosen in vitro developed schizonts in relation to their number of nuclei. Schizonts with 16–20 nuclei were mature with separate merozoites. DNA contents were established as in Fig. 2. Straight lines indicate expected values, either when all nuclei within a cell were haploid (lower line) or when all were diploid (upper line).

Addition of aphidicolin to in vitro cultures had no influence on trophozoite development. At 15 h of incubation (18 h p.i., i.e. 15-18 h after invasion) asexual parasites in all wells, including those containing aphidicolin, had developed into large trophozoites, as in vivo. During the next 6 h nearly all trophozoites in the controls (± 90%) developed into mature schizonts and

so did the trophozoites when the aphidicolin was removed by washing (Fig. 5). The presence of aphidicolin during schizogony, however, inhibited DNA synthesis and mitosis drastically, independently of whether the trophozoites in the preceding 15 h period of development had or had not been incubated with the drug; 50% inhibition was achieved at about 30 μM aphidicolin (Fig. 5).

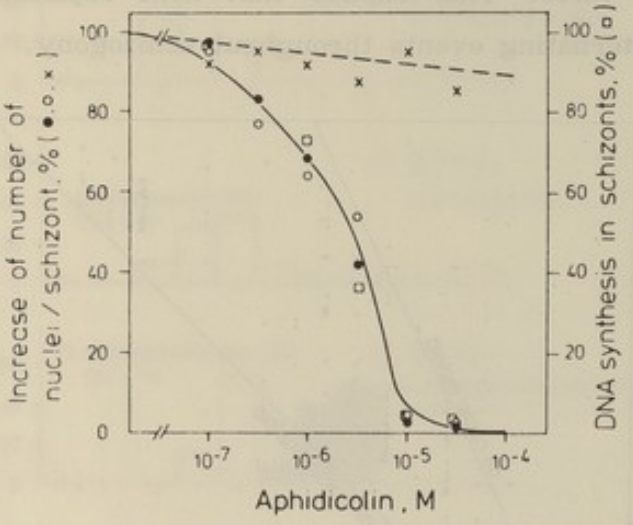

Fig. 5. Effect of aphidicolin on in vitro schizogony. Ring-forms (0–3 h after invasion) were incubated for 21 h at 37°C in RPMI 1640 at pH 7.3 in 24 well plates with various aphidicolin concentrations. (1) At 21 h of incubation the mean number of nuclei per schizont in 3 wells of each aphidicolin concentration was established in Giemsa-stained smears (100 parasites per well). The increase of the number of nuclei per parasite in controls was on average 11.4 (= 100%). (•) Parasites incubated with aphidicolin for 21 h. (○) Parasites incubated without aphidicolin for the first 15 h after which aphidicolin was added. (x) Parasites incubated with aphidicolin for the first 15 h after which aphidicolin was removed. (2) At 21 h of incubation the mean DNA content per schizont was established as in Fig. 2 in 2 wells of each concentration (100 parasites per well). The increase of DNA content per parasite in controls was on average 1358 F.U. (= 100%). (□) Increase of DNA in parasites incubated with aphidicolin for 21 h.

Gametocytes. At 26 h p.i. (during the increase of capacitated microgametocytes) and at 35 h p.i. (during the period of stable numbers of capacitated microgametocytes) both gametocytes had DNA values between the haploid and diploid value (Fig. 3d, e). No significant

differences were observed between DNA contents at 26 and at 35 h p.i. The mean amount of DNA in excess of the haploid value was significantly higher ($P<0.05$) in microgametocytes than in macrogametocytes.

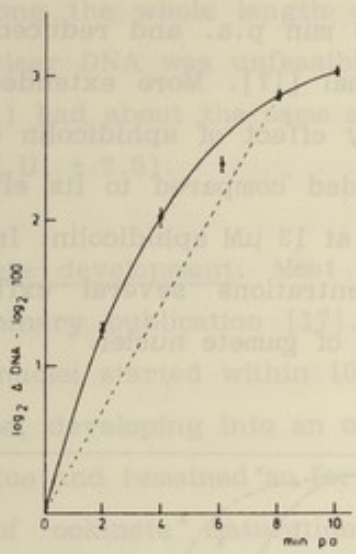

Fig. 6. The rate of DNA synthesis during microgametogenesis of *P. berghei*. The increase of DNA (Δ DNA) relative to the haploid amount (100 F.U.) is given on a $\log_2$ scale. Data represent means ± S.E.M. and are calculated from the F.U. measured in randomly chosen microgametocytes at 2 min intervals p.a. [17]. The slope of the broken line, the mean rate of increase between 0 and 8 min p.a., is used for the calculations of the number of replication origins (see text).

Gametogenesis. During gametogenesis the morphology of macrogametocytes did not change drastically except that the parasites escaped from their host cell. In microgametocytes the nucleus migrated to the middle of the parasite. At 7-10 min post activation (p.a.) the nucleus was centrally located, enlarged, rounded off and surrounded by a narrow rim of cytoplasm. Exflagellation started at 11 min p.a. in these experiments and at 13 min p.a. about 90% of the mature microgametocytes exflagellated.

The DNA content of macrogametocytes remained constant during gametogenesis, but in microgametocytes the DNA rapidly increased from less than the diploid value before activation to more than the octoploid value at 10 min p.a., just before exflagellation started [17], the highest

rate of DNA synthesis occurring during the first minutes of activation (Fig. 6).

Aphidicolin inhibited this DNA synthesis, as well as exflagellation reversibly, though morphological development of microgametocytes (apart from reduced exflagellation at 13 min p.a. and reduced Feulgen-stainability of the nucleus) appeared normal [17]. More extended investigations showed (Fig. 7) that the inhibitory effect of aphidicolin on exflagellation is less regular and somewhat retarded compared to its effect on DNA synthesis, which was inhibited for 50% at 13 μM aphidicolin. Indeed, at the moderately inhibiting aphidicolin concentrations several exflagellating microgametes often seemed to be deprived of gamete nuclei.

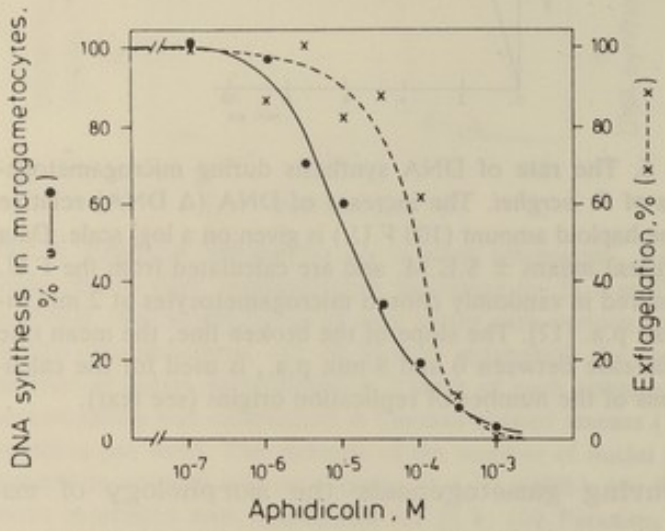

Fig. 7. Effect of aphidicolin on DNA synthesis in microgametocytes of *P. berghei* during gametogenesis ahd on exflagellation. Mature microgametocytes were incubated in vitro in microwell plates at 20°C in RPMI 1640 at pH 8.0 in the presence of various concentrations of aphidicolin and at 0 min p.a. (controls) or 10 min p.a. their DNA content was measured. Mean increase of DNA content per parasite in controls was 716 F.U. (= 100%). The number of exflagellations was established at 13 min p.a. The mean number of exflagellations per 10^5 r.b.c. in the controls was 1433 (= 100%).

Pre-incubation of mature gametocytes with aphidicolin at 37°C in RPMI 1640 at pH 7.3 for 2-6 h had no influence on exflagellation or DNA synthesis

after the cells had been transferred to RPMI 1640 at pH 8 without aphidicolin (data not shown).

Only a few free microgametes could be measured, because nearly all microgametes showed such a high background fluorescence (the origin of which is not clear) along the whole length of the gamete that accurate measurement of the nuclear DNA was unfeasible. The few which could be measured reliably (n=11) had about the same amount as young ring stages (mean ± S.E.M. = 103 F.U. ± 2.5).

Fertilization and ookinete development. Most of these results have been published in our preliminary publication [17]. In short, fertilization and fusion of both gamete nuclei started within 10 min after exflagellation. In less then 3 h the zygote, developing into an ookinete, synthesized DNA to about the tetraploid value and remained so for the next 21 h (the maximum period investigated) of ookinete maturation. Feeding of the mature ookinetes via a membrane [27] to susceptible A. atroparvus resulted in a 100% infection rate (10-200 oocysts per mosquito, n=28). In zygotes 50% inhibition of DNA synthesis occurred at 10 μM aphidicolin.

Discussion and Conclusions

Haploid stages. Young ringforms have the same amount of DNA - equal to that of microgametes - and the same symmetrical frequency distribution of DNA values as sporozoites, showing that both, as expected, are haploid non-DNA-replicating stages. This confirms (1) that meiosis occurs during development in the mosquito, as is generally accepted [28] and (2) since clones derived from asexual bloodforms can produce both micro- and macrogametocytes [29, 30], that sexual dimorphism in Plasmodium, like in other Eucoccida [31], is a phenotypic differentiation.

Schizogony. Our results confirm earlier observations on the time of DNA synthesis during asexual development (see Introduction): DNA synthesis

starts in large trophozoites shortly before the onset of nuclear division. Moreover, we found that each replication of the genome is immediately followed by segregation of both haploid chromosome complements into two light-microscopically distinct chromatin aggregations. Total schizogony took 4-6 h, in which 16-20 daughter nuclei were formed, which equals to an average of more than 1 h for each nuclear duplication.

Gametocytogenesis. Both mature gametocytes had DNA amounts between the haploid and diploid value. Most, if not all of the cells measured at 22 h p.i. (Fig. 3c, left) must be developing gametocytes (see Results: trophozoites and schizonts). Their mean DNA value is already significantly above the haploid value ($P<0.01$). It follows that DNA synthesis in gametocytes starts before 22 h p.i. Whether the DNA in excess of the haploid value reflects an onset of normal genome replication or selective gene amplification, as has been suggested to occur in young microgametocytes and macrogametes, respectively, of I. (T.) gondii [16], remains to be determined. From our results, however, it is unlikely that a complete genome duplication occurs during the development of macrogametocytes of P. berghei, as has been concluded to occur in P. falciparum from the killing effect of mitomycin-C on very young macrogametocytes [32] and from the finding of intra-nuclear microtubules in this stage [9]. Our own experiments with mitomycin-C [26] demonstrated that this is an unreliable drug to study DNA synthesis in Plasmodium. In our view selective gene amplification is the more likely explanation for the observed excess of DNA in macrogametocytes of P. berghei (and probably in microgametocytes too) since the same amount of extra DNA as in mature macrogametocytes is found in macrogametes and in young zygotes. It has been suggested that amplified DNA in these stages might code for ribosomal RNA, but no evidence of rDNA amplification in macrogametocytes of P. chabaudi and P. berghei has been found [33].

Microgametogenesis; replication origins. The most remarkable result of the present study is the very rapid DNA synthesis during microgametogenesis.

We calculated from the mean rate between 0 and 8 min p.a. (dashed line in Fig. 6). that the entire haploid genome is replicated in, on average, 3.2 min. Assuming the rate of fork movement in Plasmodium to be equal to that in other eukaryotes (about 50 base pairs (bp) s^{-1} or 9.6 kilo base pairs (kbp) in 3.2 min), it follows from the most accurate estimation available of the haploid genome size of P. berghei (27 fg=25 Mega base pairs (Mbp) [16]) that there must be at least 1300 (25000/(2 x 9.6)) origins of replication with a mean distance of at most 2 x 9.6 kbp (6.4 μm), short enough to be visible at the electron microscope level in preparations made according to the Miller's spreading technique [34] or of purified DNA extracted from exflagellating microgametocytes. The high number of replication origins in the relatively small Plasmodium genome may account for part of the repetitive DNA fraction in DNA extracts of this parasite as has been suggested by Guntaka et al. [35] in the case of P. falciparum.

Zygote development. The time of DNA synthesis in zygotes coincides with the earliest time at which synaptonemal complexes are found [15], which strongly suggests that this DNA synthesis represents the genome replication of the first meiotic division. We have at present no satisfying explanation why mature ookinetes have slightly but significantly lower than the expected tetraploid value, apart from assuming that most zygotes have not yet completed their premeiotic replication at 24 h p.i.

DNA-polymerase of Plasmodium. An α-like, aphidicolin sensitive DNA polymerase has been found in all kinds of eukaryotic organisms from higher plants and vertebrates to fungi and protozoa [36] and Plasmodium apparently is no exception to the rule. We observed a 3-4 fold higher C_{50} value of aphidicolin inhibition in microgametocytes and zygotes than in schizonts, which may reflect different levels of DNA polymerase or desoxyribonucleoside triphosphates, as has been found in mutants a of Drosophila melanogaster cell line [37] and in mutants of a hamster cell line [38] and of the eucoccidian parasite I. (T.) gondii [39], respectively.

Nevertheless, even the lowest value found in P. berghei, which agrees with that inhibiting schizogony of P. falciparum [6] and DNA synthesis in the RH strain of the related coccidian I. (T.) gondii (2.7 μg ml $^{-1}$ = 8 μM; see ref. 39), is considerably higher than the C_{50} value found in vivo in other eukaryotes, including mammals [22, 23, 25, 37-41]. The enzyme of Plasmodium, therefore, may be slightly different from mammalian DNA polymerase-α, as was also found in Trypanosoma brucei [42]. If this were the case, it would justify the search for a specific inhibitor, which could open a new approach in immunization against malaria (and possibly other protozoan infections) focussed on reducing the parasite's multiplication rate and in this way enabling the host to build up his own immunity. An advantage of this approach would be that a Plasmodium-specific DNA-polymerase, unlike antigens, can be expected to show less geographic and other intraspecific variation, in view of the conserved nature of DNA-polymerase genes [36].

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Chapter IX

DNA SYNTHESIS IN GAMETOCYTES OF PLASMODIUM FALCIPARUM

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Summary

DNA contents of P. falciparum gametocytes during intra-erythrocytic development and during gametogenesis were established by cytophotometric methods.

Intra-erythrocytic micro- and macrogametocytes (Stage I till Stage VB) contain about twice the DNA amount of haploid sporozoites and ringstages, indicating DNA synthesis during transformation of ringforms into stage I gametocytes. Microgametocytes, after activation at pH 8 rapidly duplicate their genome several times, while the DNA content of macrogametocytes remains constant during gametogenesis.

Introduction

Sexual development in malaria parasites starts when a haploid intra-erythrocytic merozoite differentiates into a micro- or macrogametocyte. It is continued after ingestion of gametocytes by a vector mosquito: the macrogametocyte transforms into a macrogamete, which can be fertilized by one of the eight gametes which are produced by the microgametocyte during gametogenesis.

Recently we established the DNA contents of gametocytes of the rodent malaria parasite Plasmodium berghei (Janse et al., 1986a,b). The mature micro- and macrogametocyte in the blood of the vertebrate host have a DNA content between the haploid and diploid value, suggesting that the DNA in excess of the haploid value is the result of selective gene amplification, occurring several hours before maturity of the gametocytes. In order to produce the eight gametes, the microgametocyte after activation duplicates its genome repeatedly and at a high rate. These observations are contradictory to results of electron microscope studies on gametocytes of the human parasite P. falciparum, demonstrating the occurrence of intranuclear spindles, which suggested that micro- and macrogametocytes replicate their entire genome during development within the erythrocyte (Sinden, 1982). In macrogametocytes this replication was assumed to be followed by cryptomitosis in order to produce haploid cells, while repeated replication in microgametocytes during maturation in the blood cells was considered to result in octoploid cells (Sinden, 1983).

The measurements of DNA contents of gametocytes of P. falciparum, presented in this paper, show that in young micro- and macrogametocytes of P. falciparum DNA is synthesized to twice the haploid amount. Repeated genome duplication in microgametocytes, however, occurs only after activation.

Materials and methods

Intra-erythrocytic ringforms and gametocytes of P. falciparum (isolate NF54, Amsterdam Airport strain) were obtained from cultures in which parasite development had been synchronized (Ponnudurai et al., 1986) by a combination of gelatin flotation and N-acetyl glucosamine treatment. Sporozoites were obtained from salivary glands of Anopheles stephensi, dissected 14 days after membrane feeding of cultured gametocytes.

The relative DNA contents of sporozoites, ringforms and gametocytes were measured by direct microfluorometry of Feulgen-pararosaniline stained cells, using the same procedures and equipment as Cornelissen et al. (1984) and Janse et al. (1986b). This method allows determination of the DNA content of individual cells which can be identified morphologically. In addition to this method we established the fluorescence intensity of Hoechst-33258 stained ringforms and gametocytes by flow cytometry using a FACS Analyzer, as described by Janse et al. (1986). This method allows rapid determination of the relative DNA content of a large number of cells. The mean fluorescence intensity of ringforms was taken to represent the haploid amount of DNA (see Results and Janse et al., 1986b) and has been used as reference value in all experiments.

In vitro activation and counting of the number of exflagellating microgametocytes were performed as described earlier (Janse et al., 1985). The different gametocyte stages could be readily recognized under the microscope after Feulgen staining by size and shape of cells, nuclei and pigment granules; they were classified as described by Ponnudurai et al. (1986).

Results

Sporozoites, ringforms. After Feulgen-pararosaniline staining fluorescence values of ringforms showed a symmetrical frequency distribution with a

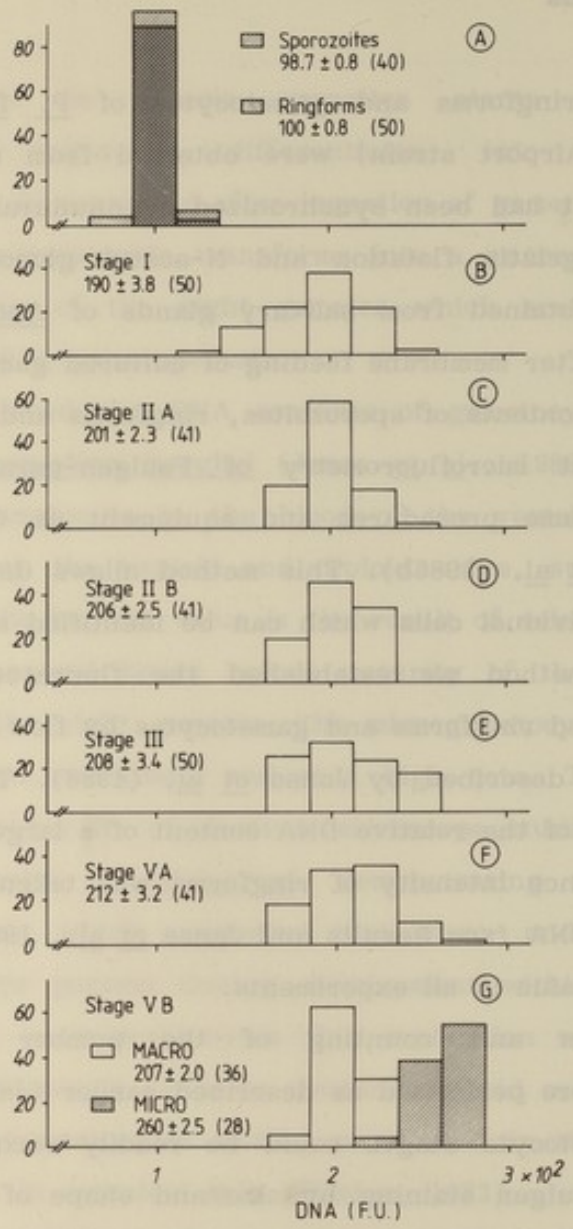

Fig.1 Frequency distributions of DNA contents of sporozoites and ringforms (a) and of developing gametocytes (b-g) of P. falciparum. DNA contents were established by microfluorometry of Feulgen-pararosaniline stained cells and expressed in arbitrary fluorescence units (F.U.). The mean value of ringforms was set to 100 F.U. Gametocytes were classified as described by Ponnudurai et al. (1986).

mean value which is equal to that of sporozoites (Fig. 1). This value is considered to represent the haploid amount (1C) of DNA.

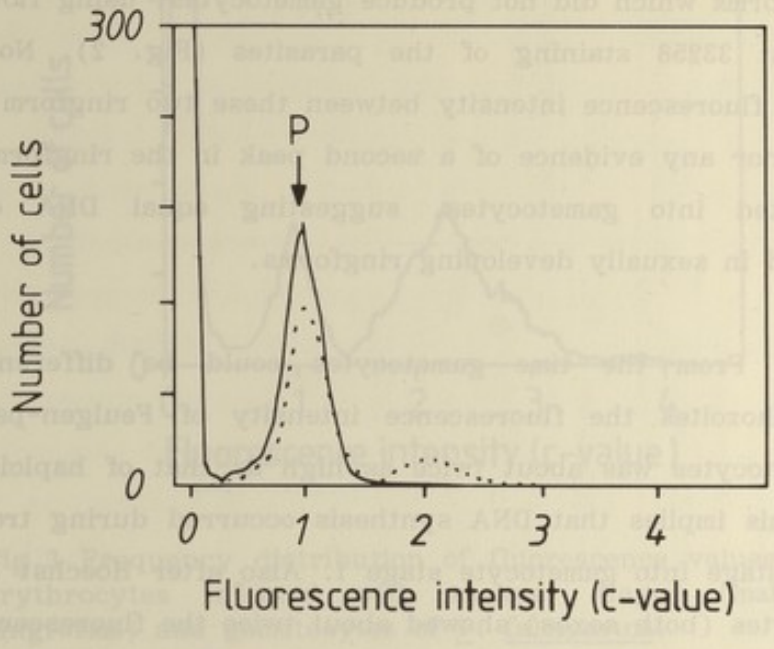

Fig.2 Frequency distributions of fluorescence values of erythrocytes infected with ringforms of P. falciparum. The fluorescence intensity was measured by flow cytometry after Hoechst-33258 staining of the cells (see for details Janse et al., 1986d)

—ring-infected erythrocytes (n=3580) of the NF54 isolate obtained from a synchronized culture in which the conversion rate into gametocytes was 20% (Ponnudurai et al., 1986). No double infected cells were present. The peak value (p) of these cells, corrected for the "back-ground" fluorescence of uninfected erythrocytes, is taken to represent the haploid value (= 1.0 C).

- -ring-infected erythrocytes (n=3107) of the NF54 isolate obtained from a synchronous culture in which no gametocytes were produced. The first peak, corrected for the background fluorescence, lies at 1.04 C. The small "second" fluorescent peak represents double infected erythrocytes (20% of infected erythrocytes).

If sexually determined ringforms should exist, these cells could have been missed in the Feulgen histogram of Fig.1, considering the restricted number of cells that can be measured by this method and the low percentage of ringforms that develops into gametocytes. Therefore, we compared the

fluorescence intensity of a large number of ringforms from a synchronous culture in which the conversion rate into gametocytes was high (20%), with that of ringforms which did not produce gametocytes, using flow cytometry after Hoechst 33258 staining of the parasites (Fig. 2). No significant difference in fluorescence intensity between these two ringform populations was found, nor any evidence of a second peak in the ringform population that converted into gametocytes, suggesting equal DNA contents in asexually and in sexually developing ringforms.

Gametocytes. From the time gametocytes could be differentiated from asexual trophozoites the fluorescence intensity of Feulgen-pararosaniline stained gametocytes was about twice as high as that of haploid ringforms (Fig. 1). This implies that DNA synthesis occurred during transformation of the ring stage into gametocyte stage I. Also after Hoechst 33258 staining gametocytes (both sexes) showed about twice the fluorescence intensity of haploid asexual stages (Fig. 3). At maturity microgametocytes showed a higher value (2.6C) than macrogametocytes (2.0C)(Fig 1).
Twelve minutes after activation just before the onset of exflagellation, the fluorescence intensities of microgametocytes had increased to values ranging from 4 to 12 times the haploid value, while the fluorescence intensities of activated macrogametocytes were all similar and equal to that of macrogametocytes before activation (Fig.4). These results indicate that in microgametocytes repeated genome duplication occurred during gametogenesis.

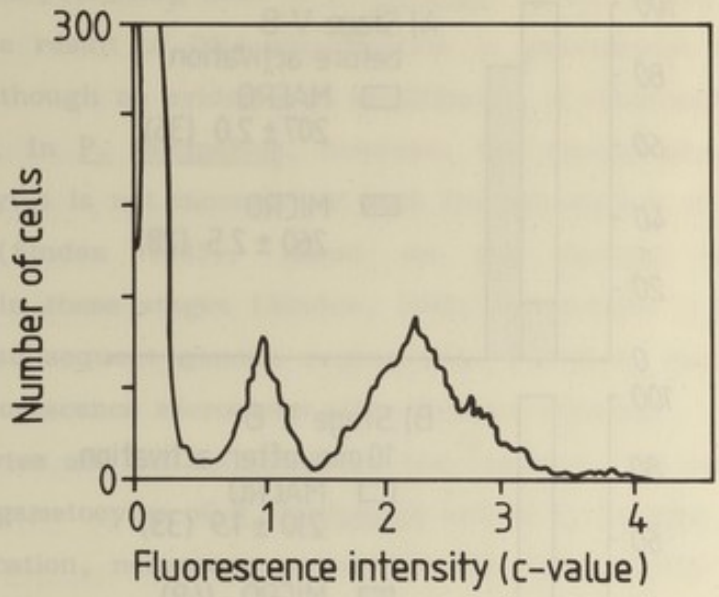

Fig.3 Frequency distribution of fluorescence values of erythrocytes infected with asexual stages (mainly ringforms) and gametocytes of P. falciparum.
Measurement of the fluorescence intensity was performed as described in Fig. 2.
Erythrocytes were obtained from an asynchronous culture and parasites had been partially purified by Nycodenz gradient centrifugation. After purification 7% of the erythrocytes contained asexual stages and 18% gametocytes (stage II - VA). These figures correspond to the flow cytometric counts: 6.3% of the erythrocytes showed fluorescence values between 0.5 and 1.5C (haploid ringforms) and 15.1% between 1.5 and 3.5C (gametocytes)

Discussion

Mature intra-erythrocytic gametocytes of P. falciparum contain more DNA than the haploid sporozoites and ringforms, as has been demonstrated for the mature gametocytes of the rodent parasite P. berghei (Janse, et al., 1986a,b). Results presented in this paper show that in P. falciparum the DNA in excess of the haploid amount is synthesized during development of ringforms into stage 1 gametocytes. The exact time of DNA synthesis in P. berghei gametocytes is difficult to establish since immature gametocytes

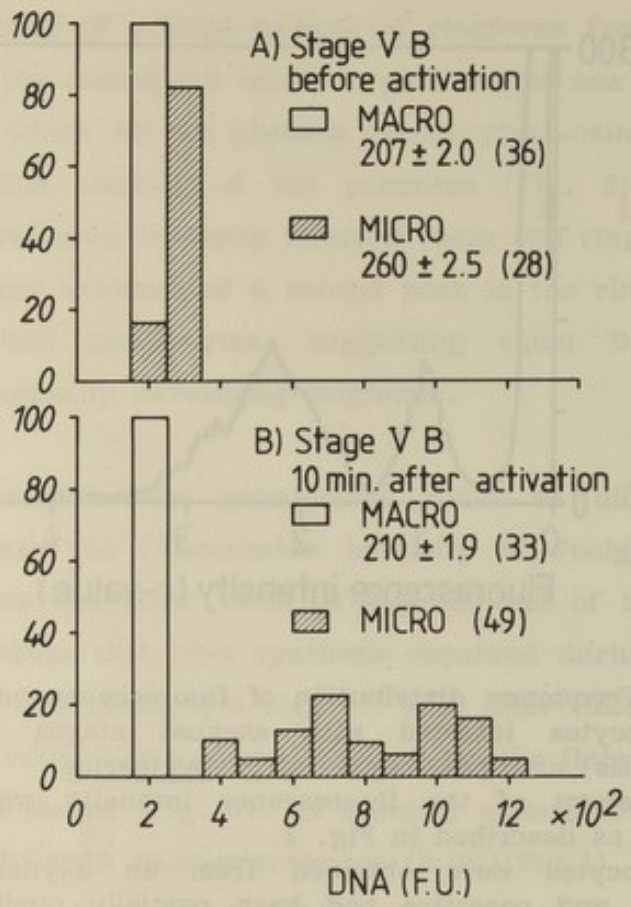

Fig.4 Frequency distributions of DNA contents of mature micro- and macrogametocytes (stage VB), measured by microfluorometry as in Fig. 1 just before (a) and 12 min after (b) activation at 20°C in RPMI 1640 medium at pH 8. Gametocytes were obtained from a synchronized culture in which 30% of the mature microgametocytes were able to undergo exflagellation, which started between 13 and 25 min after activation. In (b) only those activated gametocytes were measured which had escaped from the host cell or had rounded up.

cannot be distinguished morphologically from asexual trophozoites (Mons et al., 1985). The DNA contents of mature micro- and macrogametocytes of P. berghei are significantly below the diploid amount. We, therefore, suggested that the DNA in excess of the haploid amount is most likely the result of DNA amplification instead of replication of the entire haploid genome. Consistent with this view is the assumption that the difference in the amount of repetitive DNA between gametocyte producing and

non-gametocyte producing lines of P. berghei, as was found by Dore et al. (1980), is the result of DNA amplification in gametocytes (Cornelissen et al., 1985), although no evidence of amplification of ribosomal DNA could be demonstrated. In P. falciparum, however, the diploid amount of DNA in most gametocytes is not inconsistent with the assumption of a total genome duplication (Sinden 1983), based on the finding of intranuclear microtubules in these stages (Sinden, 1982; Ponnudurai et al., 1986). No evidence of subsequent genome segregation, however, could be observed under the fluorescence microscope after Feulgen staining.

Microgametocytes are not octoploid; on the contrary, the results show that both in microgametocytes of P. falciparum and of P. berghei rapid repeated genome duplication, necessary to produce the eight haploid gametes, does occur after activation. Earlier (Toyé et al, 1977), DNA synthesis in microgametocytes has been considered absent during gametogenesis since gamete formation proceeded normally in the presence of mitomycin-C, an inhibitor of DNA synthesis. DNA synthesis in malaria parasites, however, has shown to be highly insensitive to mitomycin-C (Janse et al., 1986c).

DNA synthesis during microgametogenesis in P. berghei has shown to proceed synchronously, up to octoploid or slightly higher values. In P. falciparum the DNA contents of microgametocytes ranged between 4 and 12 C at the onset of exflagellation. This relatively large variation in DNA values can be explained by a less synchronous course of gametogenesis in our P. falciparum experiments; only 30% of the mature microgametocytes underwent exflagellation, starting between 13 and 25 min after activation, while in P. berghei about 90% of the gametocytes started exflagellation between 11 and 14 min after activation.

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Chapter X

MITOMYCIN-C IS AN UNRELIABLE INHIBITOR FOR STUDY OF DNA SYNTHESIS IN PLASMODIUM

C.J. Janse, P.F.J. van der Klooster, H.J. van der Kaay, M. van der Ploeg and J.P. Overdulve. 1986.
Molecular and Biochemical Parasitology, 20, 33-36

Summary

Cytophotometric studies on DNA synthesis during asexual and sexual development of Plasmodium berghei contradicted earlier conclusions on DNA synthesis in Plasmodium, which were largely based on experiments in which mitomycin-C had been used as a DNA replication inhibitor. Therefore, the effect of mitomycin on intra-erythrocytic asexual development and on microgametogenesis, fertilization and zygote/ookinete development of P. berghei was studied in vitro. All DNA-synthesizing stages (schizonts, exflagellating microgametocytes and zygotes) and also DNA synthesis itself in these stages, are totally unaffected by mitomycin concentrations 10 times higher than that which inhibits normal development of the non-DNA-synthesizing rings and trophozoites. The results are explained by the mode of action of mitomycin.

Introduction

Recently we studied DNA synthesis in Plasmodium berghei by direct cytophotometry of successive stages and by investigating the effect of aphidicolin, a specific inhibitor of eukaryotic DNA-polymerase-α (1, 2). The results, especially with respect to DNA synthesis in sexual stages, were in contradiction with earlier conclusions on DNA synthesis in Plasmodium (3-6), largely based on experiments in which mitomycin-C had been used as a DNA replication inhibitor (7-9). Mature microgametocytes had been considered octoploid as a result of repeated genome replication during early development, and microgametogenesis (which occurs within a few minutes after ingestion of mature microgametocytes by a vector mosquito), had been assumed to be restricted to mitotic segregation of the eight preformed haploid microgamete complements, without actual DNA synthesis. Our investigations, on the contrary, showed evidence of rapid, repeated aphidicolin-sensitive DNA synthesis during microgametogenesis. This discrepancy prompted us to a closer study of the effect of mitomycin-C on asexual and sexual development of P. berghei. It showed that mitomycin-C is a very unreliable inhibitor for study of DNA synthesis in Plasmodium.

Material and Methods

All materials for and methods of in vitro cultivation of P. berghei (ANKA-strain) and DNA measurements were the same as in Janse et al. (2).

To study the effect of mitomycin-C (Hoechst) on intra-erythrocytic trophozoite development and schizogony, blood was withdrawn from rats with synchronized infections (10) at 3h after inoculation of these rats (0 - 3h after invasion of merozoites). It was incubated in RPMI 1640 at pH 7.3 and 37°C, either in the constant presence of mitomycin (Table 1), or without mitomycin for the first 15h after which mitomycin was added for the next 6h (Table 2). At 21h of incubation blood films were made and

stained with Giemsa to establish the relative prevalence of mature (16 to 20 nuclei) and immature (2 to 15 nuclei) schizonts and trophozoites (1 nucleus).

To study the effect of the drug on microgametogenesis and zygote development mature gametocytes were taken from infected mice during the period of constant high gametocytaemia (2). They were either not pre-incubated, or pre-incubated for 2 to 6h in RPMI 1640, with or without 10 µg/ml mitomycin, at pH 7.3 and 37°C and then, if relevant after washing with medium to remove the drug, transferred to RPMI 1640 with or without 10 µg/ml mitomycin at pH 8.0 and 20°C (for details see Table 3). At about 10 min after transfer to the higher pH and lower temperature medium (post activation: p.a.), i.e. just prior to exflagellation, the relative DNA content of activated microgametocytes was measured, and the relative DNA content of ookinetes at 24h p.a.

The number of exflagellations/10^5 r.b.c. and the ookinete yield were established as in Janse et al. (11).

Results

1. Effect of mitomycin on trophozoite development and schizogony. In the presence of drug for 21h normal development of trophozoites and schizonts was retarded, even at the lowest drug concentration, and parasites were progressively impaired and at an earlier stage with higher drug concentrations (Table 1). When drug was only present during the last 6h of incubation, reduced schizogony was only evident at the highest mitomycin concentration used (Table 2).

2. Effect of mitomycin on microgametogenesis, fertilization and ookinete development. When mature gametocytes were suspended immediately after blood sampling in RPMI 1640 at pH 8.0 and 20°C, that is without pre-incubation at pH 7.3 at 37°C, the numbers of exflagellations and the numbers of mature ookinetes at 24h p.a. in medium containing 10 µg/ml

TABLE I

The effect of mitomycin-C on in vitro trophozoite development and schizogony of *P. berghei*[a]

Mitomycin concentration ($\mu g\ ml^{-1}$)	Percentage of total of stages with			Remarks on morphology	
	16–20 nuclei	2–15 nuclei	1 nucleus	trophozoites	schizonts
0	75	24	1	normal	normal
1	5	66	29	normal	normal
3	0	34	66	large but degenerated	impaired
10	0	3	97	large and small, degenerated	–
30	0	0	100	small, degenerated	–

[a] Parasites incubated for 21 h in the presence of mitomycin; estimates in duplo.

TABLE II

The effect of mitomycin-C on in vitro schizogony of *P. berghei*[a]

Mitomycin concentration ($\mu g\ ml^{-1}$)	Percentage of total of stages with			Remarks on morphology	
	16–20 nuclei	2–15 nuclei	1 nucleus	trophozoites	schizonts
0	75	24	1	normal	normal
1	75	24	1	normal	normal
3	74	25	1	normal	normal
10	72	26	2	normal	normal
30	29	66	5	normal	many degenerated

[a] Parasites incubated for 15 h without mitomycin, followed by 6 h of incubation in the presence of mitomycin; means of triple estimates.

TABLE III

The lack of inhibition by mitomycin of exflagellation, fertilization, ookinete development, and DNA synthesis during gametogenesis and zygote development[a]

	Time of pre-incubation					
	2 h		6 h		4 h	
Mitomycin during pre-incubation:	+	–	+	–	+	–
Mitomycin in activating medium:	–	–	–	–	+	–
Number of exflagellations per 10^5 rbc (5 wells)	311±5.1	269±4.6	278±5.0	266±4.0	289±4.4	301±4.8
Ookinete yield (5 wells)	20±2.5	21±2.3	16±1.8	18±2.2	15±2.2	14±2.0
DNA content of microgametocytes at about 10 min p.a. (n=30)	753±14.5	806±13.8	883±18.1	833±15.5	892±12.3	867±14.5
DNA content of ookinetes at 24 h p.a. (n=50)	not done	not done	not done	not done	368± 2.5	363± 2.9

[a] Mature gametocytes were pre-incubated for 2–6 h in RPMI 1640 at pH 7.3 and 37°C in the presence (+) or absence (−) of 10 $\mu g\ ml^{-1}$ mitomycin, then washed with medium and transferred to wells with RPMI 1640 at pH 8.0 and 20°C (activating medium), with (+) or without (−) 10 $\mu g\ ml^{-1}$ mitomycin.
Figures are means ± S.E.M. DNA contents are expressed in fluorescence units (F.U.) relative to the haploid genome size of *P. berghei* (mean of ringforms = 100 F.U.; see ref. 2).

mitomycin were similar to those in medium without mitomycin (triple estimates). Equally, the DNA contents of ookinetes measured at 24h p.a. were the same in both cases: the means ± s.e.m. were 359 ± 3.3 fluorescent units (n=30) in the presence of mitomycin and 350 ± 3.3 (n=25) in its absence (100 fluorescent units = mean of ringforms= haploid genome value; see ref. (2)).

Pre-incubation of mature gametocytes in 10 μg/ml mitomycin for 2 to 6h had no effect on exflagellation, DNA synthesis during microgametogenesis, fertilization, ookinete development or DNA synthesis in zygotes (Table 3).

Discussion

Our results agree superficially with those of earlier experiments on the inhibitory effect of mitomycin-C on development of Plasmodium (7-9), in that trophozoite development, but not gametogenesis can be inhibited by mitomycin. However, we demonstrated (2) that in P. berghei there is little or no DNA synthesis during the first 15 to 18h of trophozoite development: it starts in schizonts just before the first nuclear division and then, during the next 4 to 6h, alternates with nuclear division until schizonts reach maturity (16-20 nuclei). In microgametocytes a dramatic DNA synthesis activity is found during the first 10 min after activation (microgametogenesis), while the only DNA synthesis activity in zygotes occurs during the first 2 to 3h of zygote/ookinete development. The mitomycin experiments show that the development of all these DNA synthesizing stages, and in fact DNA synthesis itself in all these stages, is totally unaffected by mitomycin-concentrations in the medium ten times higher than that which, if administered from time zero, inhibits the development of ring forms to mature schizonts. The only valid conclusion, therefore, is the opposite of that drawn earlier from experiments with the drug: mitomycin does not directly inhibit DNA synthesis in Plasmodium (not even in mature microgametocytes in which the drug, by pre-incubation of the cells for up to 6h, should have had ample time to

cross-link the DNA); hence it is a totally unreliable tool to study DNA synthesis in this parasite. The only highly mitomycin-sensitive stages are the non-DNA-synthesizing young intra-erythrocytic stages.
The different effect could depend on cell membrane permeability, or it may be due to the mode of action of mitomycin: it may only act in the cell as a DNA cross-linking bifunctional alkalyting agent after NADPH-dependent reduction of its quinone moiety (12). Probably, as stated before (11), the mitomycin-insensitive stages, in contrast to ringforms and young trophozoites, lack the required quinone reductase (13) and/or are deprived of all NADPH-generating enzyme activity of the pentose phosphate pathway and malic enzyme.

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Chapter XI

PLASMODIUM BERGHEI: COMPARISON OF FLOW CYTOMETRY AND MICROFLUOROMETRY FOR ASSESSMENT OF DNA CONTENT AND DNA SYNTHESIS

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Experimental Parasitology, in press

Summary

Fluorescence intensities were established by flow cytometry of different erythrocytic stages of Plasmodium berghei after staining of their DNA with Hoechst-33258 or Hoechst-33342. Parasites were obtained from highly synchronized infections or in vitro cultures. Most fluorescence measurements were performed using a low cost, clinical flow cytometer, equipped with a mercury arc lamp.

Plasmodium-infected cells could be readily distinguished from uninfected cells on the basis of Hoechst-DNA fluorescence and single, double and triple ring-infected cells were clearly separated. Percentages of infected cells as determined by flow cytometry correlated well with manual counts in Giemsa-stained bloodfilms. The relative fluorescence intensity of different developmental stages (merozoites, ringforms, trophozoites, schizonts and gametocytes) corresponded closely to the relative DNA content of these stages. Flow cytometry appeared to be a sensitive and rapid method to measure DNA synthesis during asexual development; a C_{50} value of 5μM of aphidicolin, a specific inhibitor of DNA synthesis, was established.

Vital staining of parasites in culture was possible with both Hoechst dyes. After removal of Hoechst-33258 normal in vitro development of the stained parasites was observed.

After Hoechst-staining the haploid ringforms of P. vivax showed slightly less fluorescence (15%) than ringforms of P. berghei and P. falciparum. No difference in fluorescence intensity was observed, however, by direct microfluorometry after Feulgen-pararosaniline staining, indicating that those species have the same DNA content.

Introduction

Flow cytometry of malaria parasites has appeared to be a sensitive and rapid method to detect, count and sort Plasmodium-infected cells after staining of their DNA with fluorescent dyes (Howard and Battye 1979; Brown et al. 1980; Saul et al. 1982; Myler et al. 1982; Whaun et al. 1983; Franklin et al. 1986). However, the use of this method to establish DNA contents of different stages and the rate of DNA synthesis in parasite populations has been hampered by asynchronicity of infections, presence of multiple infected host cells and lack of knowledge concerning the process of DNA synthesis in malaria parasites.

Recently, we were able to determine the exact time of DNA synthesis during asexual and sexual development of P. berghei, using direct microfluorometry of individual Feulgen-pararosaniline-stained parasites (Janse et al. 1986). Ringforms and developing trophozoites during the first 15 hour of development are haploid. During schizogony, starting at 18 hour after invasion of the host erythrocyte, the haploid amount of DNA is multiplied 16-20 times within 4-6 hours. At the same time limited DNA synthesis also occurs in maturing gametocytes up to 40-70% of the haploid amount.

The purpose of the present study was to compare flow cytometry and microfluorometry in assessing 1) DNA synthesis during intra-erythrocytic development and 2) genome sizes of different malaria parasites.

The dyes Hoechst-33258 and Hoechst-33342 (Loewe and Urbanietz 1974; Latt and Stetten 1976; Cesarone et al. 1979) were chosen for staining since these readily enter fixed and living cells in suspension (Arndt-Jovin and Jovin 1977) and have been reported to be non-toxic for P. berghei (Howard and Battye 1979).

The present work shows that results obtained by flow cytometry of Hoechst-stained cells fit very well with those obtained by microfluorometry in studying DNA synthesis during intra-erythrocytic development but is less suitable for comparative genome size measurements.

Materials and methods

Synchronized infections of the ANKA-strain of Plasmodium berghei in Wistar rats and short term in vitro cultures of this parasite were obtained as described elsewhere (Mons et al., 1985; Janse et al. 1984, 1986), except that cultured schizonts were separated by Nycodenz gradient centrifugation (Nyegaard & Co., Torshov, Norway). A detailed description of parasite morphology during synchronous development is given by Mons et al. (1985).

P. berghei merozoites, obtained from purified mature schizonts, were separated from (infected) erythrocytes essentially as described by Dennis et al. (1975). Merozoites were resuspended in 1-2 ml culture medium and added to erythrocyte suspensions under standard culture conditions.

P. berghei gametocytes, obtained from a synchronized infection at 28h p.i., were purified by Nycodenz gradient centrifugation: Infected blood was layered on top of a 48% (v/v) mixture of Nycodenz and culture medium (described by Janse et al. 1984), and centrifuged at 350 g for 25 min at 4°C. Cells from the interfase, about 80% gametocytes, were collected.

P. vivax ringforms were obtained from a Dutch patient with a parasitaemia of 0.3%, 7 months after his return from Nepal. P. falciparum ringforms (isolate NF54) were obtained from a synchronous culture established as

described by Ponnudurai et al. (1986). Differential parasite counts in Giemsa stained bloodfilms were made in 5 x 10^3 erythrocytes.
For direct microfluorometry thin bloodfilms were prepared, fixed, hydrolyzed and stained with Feulgen-pararosaniline(SO_2) (Chroma, Stuttgart, FRG). In each experiment 50 stained parasites were measured as described earlier (Cornelissen et al. 1984; Janse et al. 1986). For flow cytometry cells were either fixed with 0.25% glutaraldehyde in phosphate buffered saline (PBS) ($2x10^8$ cells/ml) for 10 min at 4°C, washed twice with PBS, stained with 2µM Hoechst-33258 (Janssen Chimica, Beerse, Belgium) in PBS ($2x10^8$ cells/ml) for 1h at 37°C in the dark, and analysed within 1h; or living cells were stained with H.33258 or Hoechst-33342 by adding them to in vitro cultures of P. berghei at 6h p.i. at a final concentration of 10µM. After 1.5-2h of incubation at 37°C in the dark, the medium was removed and the cells were resuspended in PBS at 4°C and analysed within 1h. To test their viability after Hoechst staining and incubation in PBS, about 10^6 stained parasites were i.p. injected into Swiss mice (20-25 g) or incubated in vitro under standard conditions for 20h after two washings with prewarmed (37°C) culture medium.
Hoechst-stained cells were analysed with a FACS Analyzer and a FACS IV (Becton, Dickinson, Mountain View, CA). The FACS Analyzer was equipped with a mercury arc lamp (USHIO, Japan) and UV excitation was realized using a band pass 360 and an SP 375 filter. An SP 375 was used as a dichroic mirror. The blue Hoechst fluorescence was selected with a band pass 490 and two LP 400 filters. By gating on cell volume erythrocytes and leucocytes were electronically selected for analysis, thus eliminating platelets, free parasites and cellular debris. Fluorescence intensity measurements (linear or logarithmic gain setting) were collected for 50.000 cells per sample. Fluorescence histograms, divided in 256 channels, were processed and analysed using the standard BD Consort 30 software. Cell sorting is not feasible with the FACS Analyzer. The FACS IV was operated with a high power argon ion laser with a laser emission of 20 mW in the ultraviolet (351nm/363nm). Low angle light scatter and fluorescence signals were simultaneously detected for each cell. By electronic

gating erythrocytes were selected on the basis of their forward light scatter. Further details of operating conditions have been described by Howard and Battye (1979). Fluorescence intensity measurements were collected for 50.000 cells per sample and cells of interest were electronically selected and sorted with a speed of 1000 cells/sec.

Erythrocytic ringforms contain the haploid amount of DNA (Janse et al. 1986) and were therefore used as the reference value in flow cytometry and direct microfluorometry.

Since fluorescence values of Hoechst-stained erythrocytes, infected with a single ringform, showed a narrow symmetrical frequency distribution (see Results), the peak value, after subtraction of the mean "background" fluorescence of uninfected erythrocytes, is taken to represent the mean fluorescence value of ringforms (1C=haploid value). Normally the "background" fluorescence was less than 10% the value of ring-infected cells.

Results

Ringform-infected erythrocytes, obtained at 4 and 28h after inoculation of purified schizonts, were clearly separated from uninfected cells on Hoechst-DNA fluorescence intensity (Fig. 1, 2). Percentages of infected cells, as determined by flow cytometry, correlated well with percentages established in Giemsa stained smears (Table 1, 2). Cells infected with one, two and three ringforms could be differentiated both with the FACS Analyzer and the FACS IV (Fig. 1a,f, 2 and Table 1, 2). Double and triple infected cells showed fluorescence intensities of about two and three times the fluorescence value of single infected cells, respectively. Mainly single and double infected cells were sorted from the first and second fluorescent peak respectively, as was shown by microscopic differentiation (table 2b).

The fluorescence intensity of free merozoites (mean ± s.e.m.(n): 0.96C ± 0.0004 (40,000)) was comparable to that of intra-erythrocytic ringforms.

Only a slight increase of fluorescence (± 10%) was observed during development of ringforms (4h p.i.) into large trophozoites (16h p.i.)(Fig. 1a,b;

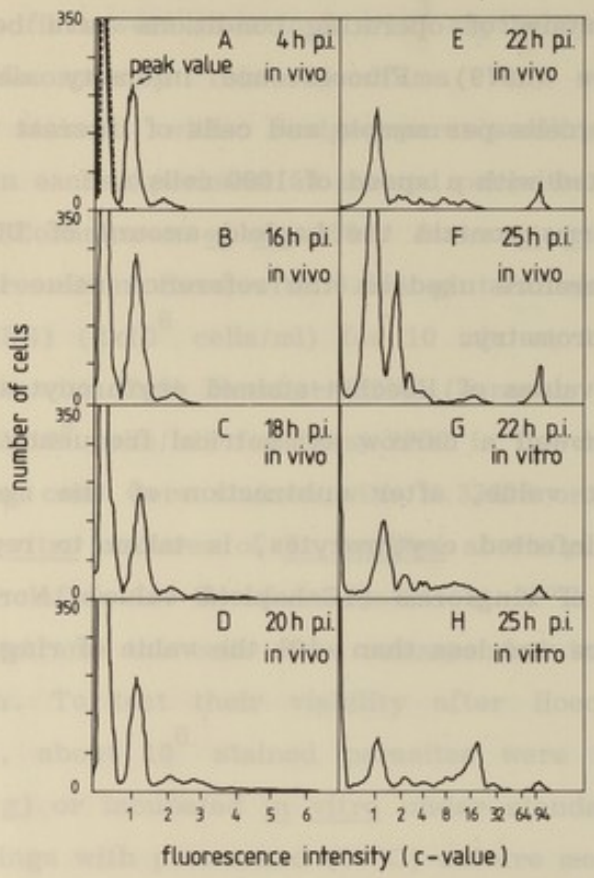

Fig. 1. Frequency distributions of fluorescence values of erythrocytes from a rat during the first 25 h of a synchronous Plasmodium berghei infection, as measured by a FACS Analyzer after Hoechst-33258 staining. The "background" fluorescence of uninfected cells was established just before intravenous inoculation of purified schizonts (see dotted line in A). At 4 hours post infection (h p.i.) in vitro cultures were started with tailblood from the rat in which only ringforms (single and double infected erythrocytes) were present. During the first 18h p.i. in vitro development is comparable to that in vivo. The haploid (1C) value is defined as the difference between the peak fluorescence value of singly infected erythrocytes (= first peak at 4h p.i.) and the mean background fluorescence. Cells with higher values than 64C were leucocytes with a mean peak value of 115C (8 exp., s.d.: 7.4). A-D: linear gain setting; E-H: logarithmic gain setting. Table 1 shows a quantitative analysis of the measured cells.

Table 1: Percentages of total number of erythrocytes of Plasmodium berghei infected cells from a synchronous infection in a rat at different hours p.i., counted in different channels when cells are measured by flow cytometry (FACS Analyzer) after Hoechst-33258 staining compared to microscopic differentiation after Giemsa staining.
In vitro cultures were started at 4h p.i. with tailblood from the rat. During the first 18h the in vitro development is comparable to that in vivo. C-values calculated as in Fig. 1.

	Flow cytometric counts % of erythrocytes (mean C-value)			Manual counts % of erythrocytes			
	channel limits			parasites per cell			
	0.5-1.5C	1.5-2.5C	2.5-40C	one	two	three	schizonts
rat							
0	0.3	0.1	0	0	0	0	0
4	6.5(1.0)	0.6(1.9)	0.1	7.0	0.5	0	0
10	6.4(1.0)	0.6(2.0)	0.1	7.0	0.5	0	0
16	6.4(1.1)	0.4(2.1)	0.1	6.8	0.7	0	0
18	5.8(1.1)	0.6(2.2)	0.4	6.3	0.5	0	0.2
20	4.4(1.2)	0.7(2.3)	0.7	3.6	0.4	0	1.0
22	4.8(1.2)	0.7(2.3)	1.6	3.8	0.5	0	2.7
25	21.2(1.0)	4.9(1.9)	2.5	23.0	5.7	0.5	0.3
culture							
22	4.3(1.2)	0.9(2.3)	2.2	3.6	0.7	0	2.7
25	4.3(1.2)	0.7(2.3)	4.0	4.1	0.5	0	4.4

Table 1). From the time the first schizonts appeared (18h p.i.) the percentage of cells with fluorescence values of over two times the haploid value increased. In vivo this percentage remained relatively low, due to disappearance of developing schizonts from the circulation and release of merozoites, which resulted in an increased parasitaemia at 25h p.i. (Fig. 1f, Table 1). In vitro, where schizonts hardly release their merozoites in the absence of shearing forces (Mons et al. 1985) the culture contained a high percentage of cells with fluorescence values of about 16C at 25h p.i. (Fig. 1h).

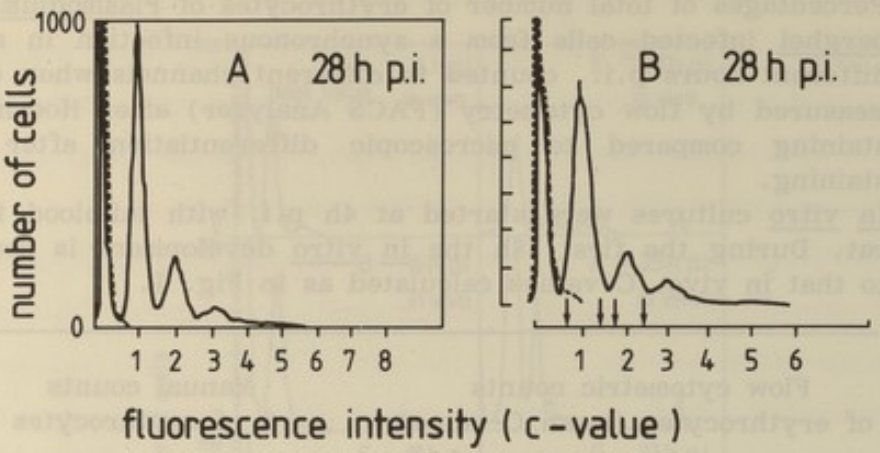

Fig. 2. Frequency distributions of fluorescence values of infected erythrocytes present in a synchronous Plasmodium berghei infection at 28h p.i., obtained with a FACS Analyzer (A) equipped with a mercury arc lamp and a FACS IV (B) equipped with a high power argon ion laser, both after Hoechst-33258 staining. At 28h p.i. only ringforms (single, double and triple infected erythrocytes) and gametocytes (less than 1%) were present. The fluorescent channels selected for sorting (0.7-1.3C and 1.7-2.3C) are indicated (see Table 2). C-values were calculated as in Fig. 1. Dotted lines show the background fluorescence of uninfected cells at 0h p.i. Table 2 shows a quantitative analysis of measured and sorted cells.

Purified mature micro- and macrogametocytes showed fluorescence values between 1 and 2C (mean $\pm$ s.e.m.(n): 1.4C $\pm$ 0.002 (36,000)), confirming the results obtained with microfluorometry of Feulgen-stained gametocytes (Janse et al. 1986)

Using flow cytometry the rate of DNA synthesis during schizogony was determined in the presence of different concentrations of aphidicolin, a specific inhibitor of DNA polymerase-α (Ikegami 1978). DNA synthesis was progressively inhibited with increasing drug concentrations and a C_{50} value of about 5μM was established (Fig. 3).

Flow cytometry was also used to study cell preference of merozoites. The experiment is outlined in Fig. 4. Invasion of erythrocytes was determined by flow cytometric and manual counts, 3h after adding merozoites to mixtures of uninfected erythrocytes from a phenylhydrazine-treated rat

Table 2: A. Percentages of total number of erythrocytes of Plasmodium berghei infected cells from a synchronous infection in a rat at 28h p.i. counted in different channels when cells are measured with a FACS Analyzer and a FACS IV after Hoechst 33258-staining, compared to microscopic differentiation after Giemsa staining.
At 28h p.i. only ringforms and gametocytes (less than 1%) were present. See Fig. 2 for frequency distributions of fluorescence values.
B. Microscopical differentation of infected erythrocytes (number of ringforms per cell) of the first and second peak (channel limits 0.7-1.3C and 1.7-2.3C, respectively, shown in Fig. 2b).

A	Flow cytometric counts % of erythrocytes			Manual counts % of erythrocytes		
	channel limits			ringforms per cell		
	0.5-1.5C	1.5-2.5C	2.5-3.5C	one	two	three
FACS Analyzer	17.3	6.0	1.6	16	5	2
FACS IV	16.9	5.5	1.4	16	5	2

B ringforms per cell	none	one	two	three
	% of sorted cells			
'first peak'(0.7-1.3C)	8	89	3	0
'second peak'(1.7-2.3C)	5	8	82	5

(mainly reticulocytes) and erythrocytes from an untreated rat. Invasion increased with an increase of the percentage of phenylhydrazine induced reticulocytes (Fig. 4), indicating that in vitro invasion of P. berghei is strongly dependent on the availability of these cells (see also Janse et al. 1984).

Living parasites - ringforms (Fig. 5), trophozoites and schizonts (data not shown) - could be stained with H.33258 as well as with H.33342. The fluorescence intensity of infected cells was dependent on the dye

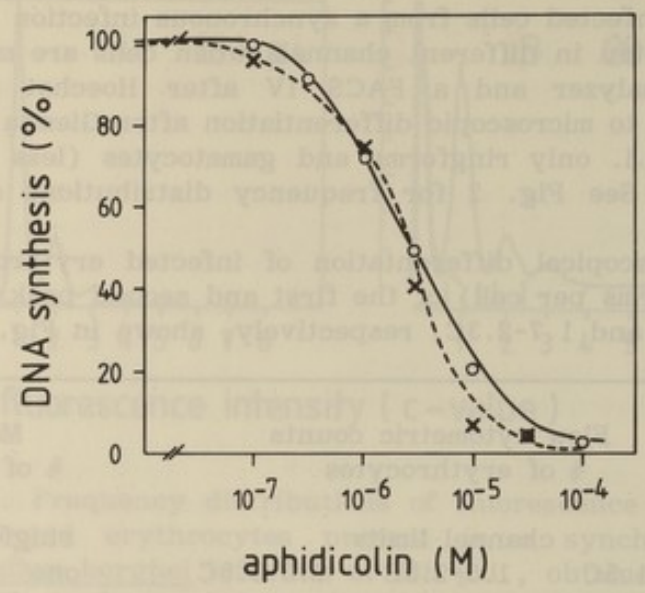

Fig. 3. Inhibition of DNA synthesis in asexual Plasmodium berghei parasites by aphidicolin, as measured by flow cytometry (FACS Analyzer) after Hoechst-33258 staining. Ringforms (4h p.i.) were incubated for 18h under standard culture conditions with various aphidicolin concentrations. At 0 and 18h of incubation the fluorescence intensity of infected cells was determined. At 0h infected cells had fluorescence values between 0.5 and 2.5C with a mean of 1.2C (single and double ring-infected cells). At 18h 70% of parasites in the controls had fluorescence values higher than 2.5C with a mean of 10.1C, while thirty percent had still values between 0.5 and 2.5C (mean 1.3), thus had not yet started DNA synthesis. At this time about 26% of the parasites in the controls were still uninucleated.
In all wells parasitaemia was constant during the culture period (7.1-7.3%). The rate of DNA synthesis is defined as the difference between the mean fluorescence value of infected cells at 18h (which is corrected for the mean value of parasites that did not start DNA synthesis at 18h in controls) and the mean fluorescence value at 0h.
0---0: flow cytometry.
x---x: direct microfluorometry (from Janse et al. 1986) for comparison.

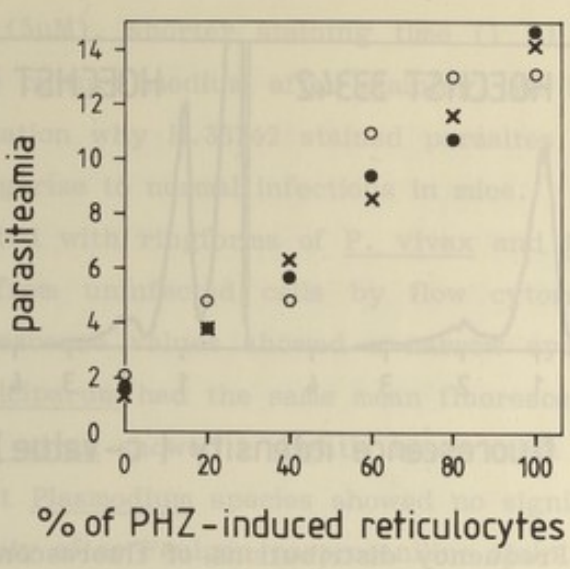

Fig. 4. Percentage of in vitro invaded erythrocytes, 3h after adding free Plasmodium berghei merozoites to uninfected erythrocytes, as determined in duplo by flow cytometry (FACS Analyzer) after Hoechst-33258 staining (●,x) and by manual counting after Giemsa staining (0).
A serial dilution of uninfected erythrocytes from a phenylhydrazine treated rat (PHZ-induced reticulocytes; 120 mg/kg body wt., 5 days prior to use) was made by mixing these cells with uninfected erythrocytes from an untreated rat and incubation under standard culture conditions in a 24 well culture plate placed on a shaker (2 wells of each dilution). To each well the same number of merozoites was added (on average 0.7 merozoite per erythrocyte). In Giemsa-stained bloodfilms only ringforms with clearly developed cytoplasm were counted to exclude free merozoites. In flow cytometric counts cells with fluorescence values between 0.5-40C were counted and a electronic gate was set on cell volume to exclude particles smaller than red cells from analysis.

concentration in the culture medium and on staining time (data not shown) as was found earlier (Howard and Battye 1979). Mice showed a normal infection pattern after inoculation of H.33258 and H.33342 stained parasites and died within 7-14 days after infection as did the control mice (6 animals/group). In vitro, H.33258-stained ringforms/young trophozoites developed normally after removal of the dye. As in control cultures 60-80% of the parasites were mature schizonts with 12-24 merozoites at 18h after

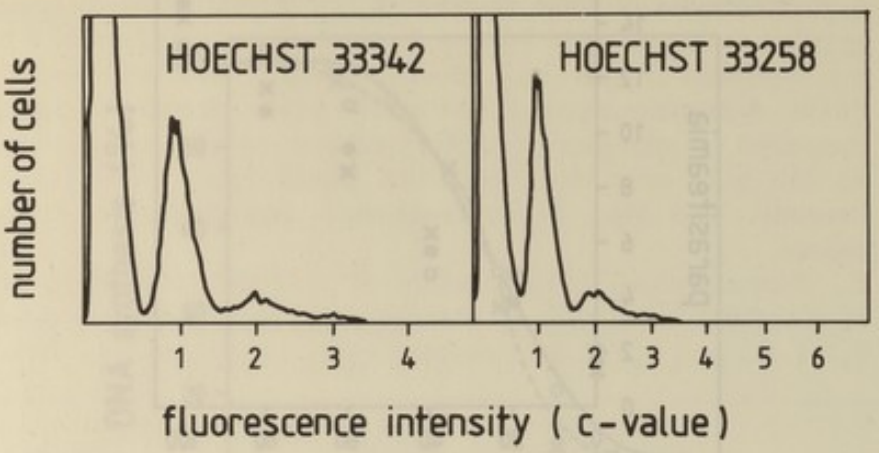

Fig. 5. Frequency distributions of fluorescence values of ringforms/young trophozoites (8h p.i.) obtained with a FACS Analyzer after vital staining with Hoechst-33258 and Hoechst-33342. Parasites were incubated under standard culture conditions in the dark for 2h with a dye concentration of 10µM. Thereafter culture medium was removed and cells were resuspended in PBS at 4°C and measured within 1h.

Table 3: Mean fluorescence value of ringforms of Plasmodium berghei, P.falciparum and P. vivax determined by flow cytometry (FACS Analyzer) after Hoechst-33258 staining and by direct microfluorometry after Feulgen-pararosaniline staining. The mean fluorescence value of P. berghei ringforms is used as reference value and set at 100 F.U.

	FLUORESCENCE INTENSITY	
	Feulgen-pararosaniline mean ± s.e.m. (n)	Hoechst-33258 mean ± s.e.m.(n)
P. berghei	100 ± 0.7 (50)	100 ± 0.15 (2500)
P. falciparum	101 ± 0.7 (50)	102 ± 0.09 (7200)
P. vivax	105 ± 1.3 (30)	85 ± 0.5 (200)

staining (3 exp.) which were capable of invasion. However, no development of ringforms was observed after H.33342 staining, even with a lower

dye concentration (5μM), shorter staining time (1 h) and more frequent replacement of the culture medium after staining. We have at present no satisfactory explanation why H.33342 stained parasites did not develop in culture while giving rise to normal infections in mice.

Erythrocytes infected with ringforms of P. vivax and P. falciparum could be distinguished from uninfected cells by flow cytometry after H.33258 staining and fluorescence values showed a narrow symmetrical frequency distribution. P. falciparum had the same mean fluorescence intensity as P. berghei whereas P. vivax showed a slightly lower value (Table 3).

The three different Plasmodium species showed no significant difference in fluorescence intensity after Feulgen-pararosaniline staining (Table 3).

Discussion

Recently we determined the DNA content of asexual and sexual developmental stages of P. berghei by direct microfluorometry of individual Feulgen-pararosaniline-stained parasites (see Janse et al. 1986 and Introduction). This staining method has been widely used in quantitative measurements of DNA, based on the well established assumption of a lineair relationship between the amount of fluorescence and the original DNA content of stained nuclei (Duijndam and Van Duijn 1975; Prenna et al. 1974; Van Prooijen-Knegt et al. 1980). Comparison of data obtained by direct microfluorometry with results presented in this paper show that the fluorescence intensities of Hoechst-stained P. berghei parasites correspond with the DNA content of these parasites. The slight increase of fluorescence intensity observed during the first 16 hour of trophozoite development, during which no DNA synthesis is expected, most probably is due to the enlargement of the parasite cytoplasm since we observed a faint fluorescence of the cytoplasm under the fluorescence microscope. From 16h p.i. onward the fluorescence intensity of asexual parasites increased to about 16 times the haploid value in mature schizonts.

Flow cytometry is an adequate method to study DNA synthesis inhibition by aphidicolin: a similar C_{50} value of about 5 μM was established as earlier found by microfluorometry (Janse et al. 1986).

In short flow cytometry showed to be a sensitive and very rapid method to determine the onset and rate of DNA synthesis during schizogony. A disadvantage, however, is that parasites which have started DNA synthesis to 2 or 3 times the haploid amount cannot be distinguished from double and triple infected cells by the FACS and it is evident that some details of the process of DNA synthesis during schizogony and gametocytogenesis, as published earlier (Janse et al. 1986) can only be determined by microfluorometry of individual cells. On the other hand, vital staining and separation of cells based on their different DNA content would make it feasible to select living cells with altered growth properties, e.g. aphidicolin-resistant cells or temperature-sensitive mutants (Arndt-Jovin and Jovin 1977).

By direct microfluorometry of Feulgen-pararosaniline-stained parasites we found no significant difference in fluorescence intensity between the haploid stages of P. berghei, P. vivax and P. falciparum, indicating that these species have the same DNA content. By comparing Feulgen-pararosaniline-stained sporozoites and metaphase human chromosome 21, this haploid genome size has been estimated in P. berghei as 2.5×10^7 bp (Cornelissen et al. 1984). After Hoechst staining we observed no difference between the fluorescence intensity of P. falciparum and P. berghei; however, P. vivax showed a small but significantly lower value. This difference may be due to the preference of Hoechst dyes to bind to A-T rich-regions of the DNA (Latt and Wollheb 1975). P. falciparum and P. berghei have the same low G-C content (18%) in contrast to the DNA of P. vivax which consists of a low and a high G-C content component (18% and 30%, respectively) (McCutchan et al. 1984). As a result we may expect a lower affinity of Hoechst dyes for P. vivax DNA.

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Chapter XII

GENERAL DISCUSSION AND CONCLUSIONS

Improvement of the P. berghei-rodent model

To study sexual development of malaria parasites, P. berghei has several advantages over other Plasmodium species. For example, optimal sexual reproduction of strains can be maintained since transmission by mosquitoes is easily performed in the laboratory. Without regular mosquito passage, strains or even cloned lines may lose their capacity to produce sexual cells (Birago et al., 1982; Mons, 1985). Another advantage is the possibility to perform experiments under fairly standardized in vivo conditions since P. berghei infects common laboratory rodents.

Study of gametocytogenesis in vivo, however, was still complicated by uncontrollable and unknown host factors (Vanderberg and Gwadz, 1980; Mons, 1986) and by the asynchronicity of parasite development during infections (Garnham, 1966). Therefore, the methods for in vitro cultivation and synchronization of erythrocytic development, described in this thesis, proved to be indispensable. These enable 1) study of sexual development under highly standardized conditions, with the possibility to combine in vitro and in vivo experiments, 2) establishment of time and rate of DNA synthesis in sexual cells and 3) purification of large numbers of various life cycle stages, which opens the way to molecular and biochemical studies of sexual stages of P. berghei. The use of these methods in this study has provided basic information about some aspects of the biology of P. berghei, particularly of gametocytes.

(1) Very recently evidence has been obtained that in P. berghei sexual commitment of intra-erythrocytic parasites takes place between 8 and 12 hours erythrocyte invasion (Mons, 1986). Sexual trophozoites, however, can not be distinguished from asexual trophozoites before 18-22 hour after invasion, both at the light- and electronmicroscope level (chapter IV and Mons, 1986), nor can they be differentiated on their DNA content (chapter VII).

Gametocytes reach maturity at about 26 hour after invasion of the merozoite and from this time onward they show sexual dimorphism (chapter

IV). The short developmental time of gametocytes of P. berghei is in marked contrast to the 7-12 days of gametocyte maturation in the human parasite P. falciparum (Hawking, Wilson and Gammage, 1971; Ponnudurai et al., 1986). This extended period of gametocyte development in P. falciparum is exceptional among Plasmodium species (Garnham, 1966).

(2) Survival time of mature P. berghei gametocytes (i.e. the time during which they are able to produce gametes) has been estimated at 26 hours. It seems that degenerating gametocytes do not persist for prolonged periods but are rapidly cleared from the peripheral circulation (Chapter IV, VIII). Nonetheless some authors described persistant circulating gametocytes, which they considered to be degenerated (Cantrell and Jordan, 1946; Eyles, 1952; Dei Cas et al., 1980).

(3) In laboratory hosts we found no evidence for differences in spatial distribution of immature or mature gametocytes, as has been described for young P. falciparum gametocytes which undergo maturation in deep tissues, and as is suggested for infective gametocytes of P. yoelii which seem to be distributed preferentially in the capillaries of the host (Landau et al. 1979). P. berghei gametocytes appeared to be randomly distributed in capillary and peripheral blood (chapter IV,V).

(4) A large proportion of morphologically mature macrogametocytes, both in synchronous or asynchronous infections, is functionally mature, i.e. capable of producing zygotes in vitro. Nearly all mature microgametocytes can produce gametes at 10-14 minutes after activation, within one hour followed by fertilization of macrogametes (chapter VIII). They will probably also do so in vivo since zygote development within the midgut is highly comparable to that in vitro. However, in mosquitoes only a small fraction of functionally mature macrogametocytes actually develops into mature oocysts (Chapter VI; Vanderberg, Weiss and Mack, 1977), probably due to factors which prevent or inhibit fertilization and oocyst development.

DNA synthesis in malaria parasites

In this study we combined the techniques for in vitro cultivation and synchronization of parasite development with microfluorometry of Feulgen-pararosaniline stained parasites to establish time and rate of DNA synthesis during asexual and sexual development in Plasmodium The advantages of measuring DNA contents on a cell by cell basis over biochemical methods have been discussed in chapter I.1. The results will only briefly be summarized and discussed below, having been discussed in more detail in the separate chapters.

DNA content and genome size. Sporozoites, microgametes and erythrocytic ringforms of P. berghei are non DNA-synthesizing stages, containing the haploid amount of DNA (chapter VII). By comparing the DNA content of sporozoites with that of the smallest human chromosome (nr 21), the haploid genome size of P. berghei has been estimated at 2.5×10^7 bp (Cornelissen et al., 1984). Our results show that the genomes of the human parasites P. falciparum and P. vivax are of the same size as that of P. berghei (chapter XI). In the light of these observations and other estimates of genome sizes in Plasmodium (chapter I.2) the more than ten times higher value (3×10^8 bp) reported for P. falciparum (Hough-Evans and Howard, 1982) must be considered an overestimation.

In rodent malaria parasites the repetitive fraction of the total amount of DNA in non-synchronized lines producing infective gametocytes is considerably higher (up to 15%) than in lines producing either poorly infective or no gametocytes (Dore et al., 1980; Birago et al., 1982; Casaglia et al., 1985). This difference was thought to be the result of selective gene amplification in erythrocytic stages of the lines which produce infective gametocytes. We did not find evidence for differences in DNA content of ringforms (table 1) and young trophozoites (results not shown) of P. berghei between lines/clones which differ in their capacity to produce gametocytes. Mature infective gametocytes of P. berghei (chapter VII, VIII) and gametocytes of P. falciparum (chapter IX), however, contain a

Table 1: DNA contents of ringforms (mean ± s.e.m.(n)) of cloned lines and strains of P. berghei*, which differ in their capacity to produce gametocytes.

STRAIN	ANKA	K173
GAMETOCYTE PRODUCTION	10 - 20%	0%
DNA CONTENT - Feulgen-p.	100 ± 0.7(50)	99.2 ± 0.6(30)
Hoechst-33258	100 ± 0.1($1x10^4$)	99.1 ± 0.14($0.5x10^4$)
CLONED LINE (from ANKA)	8417	8428
GAMETOCYTE PRODUCTION	25 - 35%	0.5 - 2%
DNA CONTENT - Fuelgen-p	100 ± 1.0(30)	100.1 ± 1.2(30)
hoechst-33258	100 ± 0.06($1x10^4$)	99.4 ± 0.07($1x10^4$)

*The lines 8417 and 8428, a low and a high gametocyte producer, respectively, have been cloned from the ANKA-strain as described by Mons (1986). The K173 strain, which has been maintained by mechanical passage for years, does not produce gametocytes. Gametocyte production is defined as the percentages of ringforms that develops into gametocytes in synchronized infections under standardized conditions (see Mons, 1986).
Ringforms have been obtained from synchronized infections, established in rats as described in chapter IV. DNA contents were determined by flow cytometry after Hoechst-33258 staining of parasites as described in chapter XI and by microfluorometry after Feulgen-pararosaniline staining as described in chapter VIII. The mean fluorescence value of ringforms of the ANKA strain and of clone 8417 are used as reference value and set at 100 F.U.

significant amount of DNA in excess of the haploid value, which may indeed be the result of gene amplification, although in P. falciparum we cannot exclude that it could also be the result of genome duplication (Sinden, 1983). The assumed gene amplification in P. berghei might be

responsible for the observed differences in the amount of repetitive DNA between lines which show varying abilities to produce sexual cells.

DNA synthesis during asexual multiplication. In asexual trophozoites of P. berghei DNA synthesis does not occur before 15 hours after invasion of erythrocytes, but starts just before the first nuclear division. During schizogony DNA replication and genome segregation are alternating events, resulting in the production of 16-20 haploid daughter merozoites within 4-6 hours. These results correspond with observations of most other workers that incorporation of radiolabelled precursors into DNA of erythrocytic malaria parasites is highest during the schizont stage (see chapter I.2).
In P. falciparum (Inselburg and Banyal, 1984) as well as in P. berghei (chapter VII, VIII) DNA replication is inhibited by aphidicolin, a specific inhibitor of DNA polymerase-α, indicating the presence of an α-like, aphidicolin-sensitive DNA polymerase, like in other eukaryotic organisms (Scovassi et al., 1980). Nonetheless, the C_{50} value of aphidicolin in malaria parasites is significantly higher than in most other eukaryotes (see ref. in chapter VIII). This suggests a slightly different DNA polymerase-enzyme in Plasmodium, as was also found in the flagellate Trypanosoma brucei (Chang et al., 1980). If this were the case, the enzyme could be a target for antimalarial drugs.
Apart from microfluorometry after Feulgen-staining DNA synthesis in Plasmodium has also been measured in this study by flow cytometry after staining with Hoechst dyes (chapter XI). The latter method is relatively simple and rapid in comparison with either the former or with labelling of DNA with radio-active precursors. It should be noticed, however, that the Hoechst-dyes bind preferentially to A-T rich regions of DNA (Latt and Wollheb, 1975). This property limits the use of these dyes in comparative DNA measurements of organisms which differ significantly in A-T/C-G ratio of their DNA, as was demonstrated in our comparative measurements of rodent and human malaria species (chapter XI).

DNA synthesis during sexual development. Mature micro- and macrogametocytes of P. berghei and P. falciparum contain considerable amounts of DNA in excess of the haploid, which may be the result of of selective gene amplification or of a single genome duplication, as already discussed.

DNA replication in microgametocytes, necessary for production of the eight haploid gamete genomes, proved to be quite different from existing ideas about this process (see Introduction and chapter I.2). Mature microgametocytes, both of P. berghei and P. falciparum, in the blood of the vertebrate host are not octoploid as was generally assumed (Sinden, 1983), but replicate their genome repeatedly and at a very high rate during gametogenesis in the mosquito midgut (chapter VII, VIII, IX). We calculated that the entire haploid genome of P. berghei is replicated in about 3 minutes. In various higher eukaryotes differences in the rate of DNA replication are possible as a result of differences in the number of activated replication origins. Assuming that DNA replication (i.e. the rate of fork movement) in Plasmodium is equal to that in other eukaryotes, we estimated that at least 1300 replication origins in the haploid genome must be simultaneously activated during microgametogenesis (chapter VIII). A high number of replication origins in the small Plasmodium genome may account for at least part of the repetitive fraction of its DNA (Guntaka et al., 1985).

Fertilization of macrogametes in vitro takes place within 1h after activation of gametogenesis. Within 2 or 3h fertilization is followed by DNA synthesis to about the tetraploid value (chapter VII, VIII). The time of this DNA synthesis coincides with the time of meiosis (Sinden and Hartley, 1985); it should, therefore, be considered to represent the genome duplication of the first meiotic division. It seems, therefore, that in Plasmodium meiosis follows the normal eukaryotic pattern and normal patterns of recombination of genes might be expected. This is in contrast to the related parasite Eimeria tenella, in which the fertilized zygote seems to move directly from a 2N organization back to a haploid genome at the first meiotic division (Canning and Morgan, 1975).

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SAMENVATTING

Malaria

Malaria wordt veroorzaakt door eencellige organismen (protozoën) van het geslacht Plasmodium, gewoonlijk malaria-parasieten genoemd. Het zijn parasieten zowel van zoogdieren als van vogels en reptielen; ze worden van de ene naar de andere gastheer overgebracht via muskieten.

In 1955 startte de Wereldgezondheidsorganisatie een grootscheepse bestrijdingscampagne met als doel uitroeiing van malaria-parasieten die de mens infecteren. Met behulp van insecticiden trachtte men populaties van muskieten, die de parasieten overbrengen, te reduceren en door verspreiding van geneesmiddelen, die de parasieten in het lichaam van de mens doden, het aantal malaria-dragers onder de bevolking terug te dringen.

Na aanvankelijk succes met de bestrijding van malaria, blijken de gebruikte methoden te falen. Factoren die hierbij een rol spelen zijn: optredende ongevoeligheid van muskieten voor de insecticiden, snel ontwikkelende resistentie van de parasieten tegen de geneesmiddelen, en een niet optimaal functionerende gezondheidszorg, met name op het platteland in ontwikkelingslanden.

Thans wordt het aantal klinische malaria-gevallen in de (sub)tropen geschat op 100 miljoen per jaar. In tropisch Afrika sterven per jaar meer dan een half miljoen kinderen aan deze ziekte.

Naast de ontwikkeling van nieuwe geneesmiddelen wordt thans in laboratoria over de gehele wereld onderzoek verricht naar alternatieve bestrijdingsmethoden van malaria-parasieten, met name naar de mogelijkheden tot ontwikkeling van een vaccin.

Levenscyclus van malaria-parasieten

Een malaria-infectie begint met de injectie van parasieten (sporozoieten) in het bloed van de gastheer tijdens een beet van een geinfecteerde vrouwelijke muskiet.

De sporozoiet dringt een levercel binnen, waar deze zich ontwikkelt en vermenigvuldigt via een mitotisch delingsproces, resulterend in de productie van duizenden nakomelingen, de lever-merozoieten. Deze merozoieten komen, na openbarsten van de levercel, in de bloedcirculatie terecht en dringen rode bloedcellen binnen.

In de rode bloedcel ontwikkelt de parasiet zich tot een trofozoiet, die het haemoglobine van de gastheercel consumeert. Deze trofozoiet kan zich ofwel asexueel ofwel sexueel ontwikkelen.

In het eerste geval produceert de trofozoiet via een mitotisch delingsproces (schizogonie) 8-32 erythrocytaire merozoieten, die na openbarsten van de gastheercel opnieuw rode bloedcellen kunnen binnendringen, waarna de cyclus zich herhaalt.

In elke cyclus zijn er een aantal trofozoieten die de tweede weg volgen; ze gaan niet door met asexuele vermenigvuldiging, maar differentiëren tot éénkernige voorloper-cellen, z.g. micro- en macrogametocyten, van de mannelijke en vrouwelijke geslachtscellen.

De gametocyten kunnen zich alleen verder ontwikkelen in de middendarm van muskieten. Binnen 10 minuten na opzuigen van geinfecteerd bloed door de muskiet, transformeert de macrogametocyt zonder kerndeling in een vrouwelijke macrogameet en produceert de microgametocyt door herhaalde kerndeling 8 beweeglijke mannelijke microgameten. Deze processen worden aangeduid met de term gametogenese.

Na bevruchting ontwikkelt de zygote zich tot een beweeglijke ookineet, die de middendarmwand van de muskiet penetreert. Op de buitenkant van de darmwand groeit de ookineet uit tot een oocyste, waarbinnen, via een mitotisch delingsproces, duizenden sporozoieten gevormd worden.

De rijpe oocyste barst open en de vrijgekomen sporozoieten bereiken via de lichaamsholte de speekselklieren van waaruit ze bij bloedzuigen van de muskiet een nieuwe gastheer kunnen infecteren.

Een schematische weergave van de levenscyclus van twee malaria parasieten die samen het hoofdonderwerp van dit proefschrift vormen is weergegeven op bladzijde 14 en 15.

Kader van het onderzoek en gebruikte methoden

Het is duidelijk dat fundamentele kennis betreffende de 'biologie' van de malaria parasieten noodzakelijk is voor de ontwikkeling van nieuwe methoden voor de bestrijding van malaria.
De complexe levenscyclus van de parasieten is uitgebreid beschreven, zowel op licht- als op electronen-microscopisch niveau. De genetische en moleculaire processen die een rol spelen bij de uitgebreide cyclische differentiatie zijn echter vrijwel onbekend, ondanks veel studies betreffende de biochemie van de parasiet en uitgebreid moleculair-biologisch onderzoek, m.n. betreffende de genen die coderen voor oppervlakte antigenen van de parasiet.
Voor genetische en moleculaire studies, die meer inzicht kunnen geven in de oorzaken van optreden en verspreiding van resistentie en die nieuwe wegen voor bestrijding van de parasiet zouden kunnen openen, was meer kennis van het genoom en de structuur daarvan tijdens de verschillende fasen van de levenscyclus dringend gewenst. Tot voor kort was over het aantal chromosomen, de ploïdie van de verschillende stadia, het tijdstip van meiose, etc. weinig bekend, voornamelijk vanwege het feit dat het chromatine van de parasieten niet condenseert tot afzonderlijk herkenbare chromosomen tijdens mitose, zodat karyogrammen niet vastgesteld kunnen worden met behulp van conventionele cytologische technieken.

Om een beter inzicht te krijgen in het genetisch materiaal van de malaria-parasieten hebben wij, met behulp van cytofotometrische technieken, de ploïdie van de verschillende stadia en het tijdstip en snelheid van DNA synthese bepaald, zowel tijdens de asexuele als de sexuele cyclus.
Anders dan met biochemische methoden, kan met cytofotometrische technieken de DNA hoeveelheid van individuele, intacte cellen nauwkeurig vastgesteld worden.
Wij hebben voornamelijk gebruik gemaakt van microfluorometrie van Feulgen-pararosaniline gekleurde cellen, waarbij de fluorescentie gemeten wordt van cellen die stuk voor stuk morfologisch herkenbaar zijn. Deze

methode is en wordt veel gebruikt in quantitatieve DNA metingen, gebaseerd op de lineaire relatie tussen de hoeveelheid fluorescentie van de aan het DNA gebonden kleurstof-moleculen en de relatieve DNA inhoud van de cellen.

Aan het begin van dit onderzoek was weinig bekend over de details van de sexuele ontwikkeling van malaria-parasieten, waardoor het verkrijgen van de juiste sexuele stadia in voldoend grote aantallen voor de DNA metingen een probleem vormde.
Gedetailleerde informatie ontbrak over 1) de relatie tussen morfologie, leeftijd, rijpheid en infectieusiteit van de geslachtelijke stadia, 2) de temporele en ruimtelijke verspreiding van deze cellen in het bloed van de gastheer, en 3) de morfologie van de parasieten tijdens gametogenese en zygote ontwikkeling.
Om meer inzicht te krijgen in bovengenoemde punten zijn daarom methoden ontwikkeld voor de in vitro kweek en synchronisatie van de sexuele ontwikkeling van de malaria-parasiet Plasmodium berghei.
Dit is een parasiet van knaagdieren die daarom, in tegenstelling tot de malaria-parasieten van de mens, in relatief goedkope proefdieren als rat en muis kan worden gehouden en dan ook wereldwijd wordt gebruikt als onderzoeksmodel. Een routinematige transmissie van deze parasiet door muskieten is eveneens mogelijk in het laboratorium, waardoor een optimale productie van geslachtelijke stadia gewaarborgd blijft en onderzoek aan de ontwikkeling van sexuele stadia in de middendarm van de muskiet mogelijk is.

Resultaten

In vitro kweek van bloedstadia

In hoofdstuk II en III is een kweeksysteem beschreven, waarin de continue in vitro kweek van bloedstadia van P. berghei gerealiseerd kan worden. Deze in vitro kweek stelde ons onder andere in staat om, onder

gestandaardiseerde omstandigheden, het tijdstip en snelheid van DNA synthese tijdens schizogonie (asexuele vermenigvuldiging) vast te stellen (zie onder). In vivo (in ratten of muizen) is schizogonie moeilijk te bestuderen omdat dit proces niet plaatsvindt in het 'perifere' bloed, maar in de moeilijk toegankelijke capillairen.

Synchronisatie van de ontwikkeling van bloedstadia

De ontwikkeling van bloedstadia van P. berghei in ratten en muizen verloopt min of meer asynchroon, met het gevolg dat de opeenvolgende stadia niet na elkaar maar tegelijkertijd in het bloed gevonden worden tijdens een infectie. Dit maakt het moeilijk om de ontwikkelings- en overlevingstijd van de verschillende stadia vast te stellen.
In hoofdstuk IV is een methode beschreven voor in vitro en in vivo synchronisatie van de asexuele en sexuele ontwikkeling van de parasiet in het bloed.
In synchrone infekties blijkt de asexuele ontwikkeling van de binnengedrongen merozoiet tot een rijpe schizont 23-24 uur te vergen, terwijl de ontwikkeling tot rijpe gametocyten ongeveer 26 uur duurt. De laatste blijven daarna ± 26 uur in staat gameten te vormen, waarna zij degenereren en verwijderd worden uit het bloed van de gastheer.
De synchrone in vivo infekties en in vitro kweken stelden ons in staat de DNA inhoud van rijpe gametocyten en het tijdstip en de snelheid van de DNA synthese tijdens asexuele ontwikkeling exact te bepalen (zie onder).

In vitro fertilisatie en zygote-ontwikkeling

Hoofdstuk V beschrijft een methode voor in vitro fertilisatie en zygote ontwikkeling van P. berghei en de licht-microscopische morfologie van de verschillende stadia. Vergaande standaardisering maakte deze methode geschikt voor het testen van de functionele rijpheid van gametocyten, dat is het vermogen tot bevruchting en daaropvolgende ontwikkeling tot zygote.

Op deze wijze konden we aantonen dat het aantal functioneel rijpe gametocyten in 'perifeer bloed' (verkregen uit staart of hart) niet significant verschilt van dat van 'capillair bloed' (bloed dat opgezogen wordt door muskieten). Door andere onderzoekers was eerder verondersteld dat de rijpe gametocyten zich ophopen in de capillairen.
Het verloop en de tijdsduur van de ontwikkeling van de zygote in vitro komt overeen met die van de zygote in de middendarm van Anopheles atroparvus muskieten (hoofdstuk VI). In vitro is het aantal macrogameten dat wordt bevrucht en uitgroeit tot rijpe ookineet echter beduidend hoger dan in de middendarm van de muskieten.
In vitro fertilisatie en zygote-ontwikkeling stelde ons ook in staat de DNA synthese tijdens de vorming van de gameten en de ontwikkeling van de zygote te bestuderen (zie onder).

Hoeveelheid DNA van malaria-parasieten

Sporozoieten, jonge bloedstadia en microgameten repliceren geen DNA en zijn haploïd (hoofdstuk VIII). De totale haploïde hoeveelheid DNA van P. berghei is klein; ongeveer 2.5×10^7 baseparen (minder dan éénhonderdste van het menselijke haploide genoom). Wij toonden aan dat het genoom van twee malaria-parasieten van de mens, P. falciparum en P. vivax, niet of nauwelijks in grootte verschilt van dat van P. berghei (hoofdstuk XI).
Volgens andere onderzoekers bevatten malaria-parasieten die infectieuse gametocyten produceren, meer repetitief DNA - mogelijk als gevolg van DNA-amplificatie tijdens de ontwikkeling van de parasieten in de rode bloedcellen - dan parasieten die dit vermogen verloren hebben. Wij vonden geen significant verschil in de totale hoeveelheid DNA van jonge bloedstadia tussen klonen en lijnen van P. berghei, welke verschillen in hun capaciteit om infectieuse gametocyten te produceren. Rijpe infectieuse gametocyten echter, bevatten een significante hoeveelheid DNA boven de haploïde waarde, hetgeen inderdaad het resultaat van gen-amplificatie kan zijn (zie onder).

DNA synthese tijdens de asexuele ontwikkeling

Nadat P. berghei parasieten een rode bloedcel binnengedrongen zijn, groeien zij in 15-18 uur uit tot grote trofozoieten, waarna de kerndeling start. DNA-replicatie in de asexuele trofozoieten vindt niet plaats tijdens de eerste 15 uur, maar start vlak voor de eerste kerndeling. Tijdens schizogonie zijn DNA-duplicatie en genoom-segregatie alternerende gebeurtenissen, resulterend in de productie van 16-20 nieuwe merozoieten in een periode van 4-6 uur (hoofdstuk VIII).
DNA-replicatie in P. berghei kan, zowel tijdens de asexuele als de sexuele ontwikkeling, geremd worden door aphidicoline, een specifieke remmer van DNA-polymerase-α (hoofdstuk VII,VII). Dit duidt op de aanwezigheid van een α-achtig, voor aphidicoline gevoelig polymerase-enzym, zoals ook in andere eukaryoten gevonden is. Om de DNA-synthese in malaria-parasieten te remmen is echter een hogere concentratie aphidicoline nodig dan in hogere eukaryoten, hetgeen zou kunnen duiden op een afwijkende structuur van het polymerase-enzym van malaria-parasieten.
In tegenstelling tot aphidicoline blijkt mitomycine-C niet of nauwelijks de DNA synthese in malaria-parasieten te beinvloeden. Een mitomycine-C concentratie, die tien keer zo hoog is als de concentratie die parasieten doodt tijdens de trofozoiet ontwikkeling (voordat DNA gesynthetiseerd wordt), heeft geen effect op het tijdstip en de snelheid van de DNA synthese tijdens schizogonie, gametogenese en zygote-ontwikkeling (hoofdstuk X).
Naast microfluorometrie blijkt 'flow-cytometrie' zeer geschikt om quantitatieve metingen te verrichten aan DNA-synthese tijdens de asexuele ontwikkeling (Hoofdstuk XI).
In flow-cytometrie wordt de fluorescentie gemeten van cellen, gekleurd met de specifieke DNA-kleurstoffen Hoechst-33258 en Hoechst-33324, met behulp van een 'Fluorescence Activated Cell Sorter' (FACS). Deze methode is relatief simpel en snel voor bepaling van tijdstip en snelheid van DNA synthese in vergelijking met microfluorometrie of met labeling van DNA met radio-actieve precursors. Bovendien is het mogelijk de cellen vitaal te

kleuren met Hoechst-33258, waardoor het mogelijk is parasieten met veranderde groei-eigenschappen te selekteren met de FACS op grond van verschillen in hun DNA hoeveelheid en daarna verder te kweken.
Opgemerkt moet worden dat de Hoechst-kleurstoffen bij voorkeur binden aan A-T-rijke gedeelten van het DNA. Deze eigenschap beperkt het gebruik van deze kleurstoffen voor vergelijkende DNA metingen van cellen welke verschillen in de AT/CG ratio van hun DNA.

DNA synthese tijdens sexuele ontwikkeling

Een haploïde parasiet die een rode bloedcel binnendringt, kan ofwel zich asexueel ontwikkelen ofwel uitgroeien tot een mannelijke of vrouwelijke gametocyt. Tot nu toe is weinig bekend over faktoren die deze differentiatie sturen. Geslachtschromosomen spelen hierbij geen rol.
In rijpe gametocyten van P. berghei kon het tijdstip waarop het 'extra' DNA (boven de haploïde hoeveelheid) gesynthetiseerd wordt niet exact vastgesteld worden, omdat jonge gametocyten niet te onderscheiden zijn van asexuele trofozoieten. In P. falciparum duurt de ontwikkeling van gametocyten veel langer, namelijk 7-12 dagen, en zijn ze al na 48 uur herkenbaar. Deze cellen blijken DNA te synthetiseren tijdens de eerste 48 uur van hun ontwikkeling, tot ongeveer de diploïde waarde (hoofdstuk IX). Of deze toename van DNA het resultaat is van selectieve gen-amplificatie of van een totale genoom-duplicatie, zoals eerder door anderen is verondersteld op grond van aanwezigheid van microtubuli in de kern van jonge gametocyten, is een punt voor verder onderzoek.

Algemeen werd aangenomen dat rijpe mannelijke geslachtscellen in het bloed van de gastheer octoploïd zijn, dat wil zeggen dat zij hun genoom reeds 8 keer gedupliceerd hebben voor de productie van de 8 haploïde gameten tijdens het gametogenese-proces, dat zich in de middendarm van de muskiet afspeelt. Deze gedachte was gebaseerd op het feit dat de DNA-synthese-remmer mitomycine-C de formatie van de 8 gameten uit de rijpe microgametcyt niet blokkeert. Daarnaast werd herhaalde duplicatie van het

gehele genoom niet mogelijk geacht binnen dit zeer kort (± 10 minuten) durende gametogenese-proces.
Wij toonden echter aan dat rijpe microgametocyten niet octoploïd zijn, maar dat zij hun DNA een aantal malen en met grote snelheid dupliceren tijdens gametogenese (hoofdstuk VII,VIII).
We berekenden dat het gehele genoom in ongeveer 3 minuten gerepliceerd wordt. In hogere eukaryoten worden verschillen in snelheid van DNA replicatie mogelijk gemaakt door verschillen in het aantal gelijktijdig geactiveerde replicatie-startpunten in het genoom. Wanneer we aannemen dat het proces van DNA-replicatie in malaria-parasieten overeenkomt met dat van hogere eukaryoten, moeten er tijdens microgametogenese tenminste 1300 replicatie-startpunten gelijktijdig geactiveerd worden in het haploide genoom.

Uit ons onderzoek bleek dat fertilisatie in P. berghei optreedt binnen 1 uur na de vorming van de gameten, resulterend in diploïde zygoten. Binnen 2 tot 3 uur na fertilisatie vindt in de jonge zygote DNA-synthese plaats tot ongeveer de tetraploïde waarde (hoofdstuk VII). Deze DNA-synthese blijkt samen te vallen met het tijdstip van meiose, hetgeen zeer recent is vastgesteld door de waarneming van synaptische complexen in de jonge zygote van P. berghei. De DNA-synthese in de zygote kan daarom waarschijnlijk beschouwd worden als de genoom-duplicatie voorafgaand aan de meiose.
Deze waarnemingen duiden erop dat in Plasmodium een meiose optreedt die overeenkomt met die in andere eukaryoten en niet een 'éénstaps' meiose, zoals voor verschillende protozoën, waaronder Plasmodium, wordt verondersteld.

Samenvattend kan gesteld worden dat ons onderzoek heeft geleid tot de ontwikkeling van een optimaal modelsysteem voor onderzoek aan de sexuele differentiatie van malaria-parasieten en tevens tot een herziening van bestaande opvattingen over de ploïdie en het tijdstip van de DNA synthese in de geslachtelijke stadia van deze parasieten.

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