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Plasmodium berghei: The Application of Cultivation and Purification Techniques to Molecular Studies of Malaria Parasites

C.J. Janse and A.P. Waters

Species of malaria parasites that infect rodents provide models for the study of the biology of malaria parasites that infect humans. In this article, Chris Janse and Andy Waters describe some of the recent advances in the cultivation and purification methodology of one of these species, Plasmodium berghei. *The improvement of these techniques, and the increasing knowledge about the molecular biology of* P. berghei *enhance the value of this particular rodent model for the investigation of many aspects of the biology of* Plasmodium.

In research aimed at developing strategies for the eradication of human malaria, various species of rodent, avian and non-human primate malaria parasites are used as laboratory models[1]. The introduction of *in vitro* culture techniques for the most important human parasite, *Plasmodium falciparum*, has greatly increased the accessibility of this parasite for studies on basic biology. Thus, for investigation of different aspects of human infection, one could question whether or not the use of non-human malaria parasites is still appropriate. *Plasmodium falciparum* appears to be phylogenetically far diverged from other species of *Plasmodium*, even from species that infect non-human primates[2]. This unexpected evolutionary position of *P. falciparum* stresses the importance of assessing the relevance of the different models[3]. We argue here that the rodent parasite, *P. berghei*, can still be used as the model of choice to understand the basic biology of malaria parasites (which is our main area of interest), with emphasis on the study of the genetic control of cell differentiation. Improved techniques for *in vitro* cultivation and mass purification of the different developmental stages of *P. berghei* are described here, and are highlighted with new insights into the molecular biology of this parasite. These advances increase the relevance of this model for molecular studies on cell differentiation, which are either not possible or less easy to perform with *P. falciparum*, as well as other human and model malarias[1].

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The *P. berghei*/rodent model

Plasmodium berghei belongs to the group of four malaria species that infect African murine rodents[4]. Despite their phylogenetic distance[2,4], the basic biology of the murine parasites closely resembles that of the other mammalian malaria parasites[5–7]. The conservation of housekeeping genes[8] and biochemical[9,10] and genetic[11] processes between mammalian parasites (and hence similarities in drug susceptibility and mechanisms of drug resistance[12,13]) provide the first justification of the use of these rodent models in malaria research. It is easy and safe to handle and manipulate any stage of the life cycle of murine malaria parasites in the laboratory, and such models often represent the only practical means towards *in vivo* experimentation. Hence, rodent parasites are still valuable models in several

crucial areas of malaria research, such as drug testing, malaria vaccine development and the study of immunopathological reactions and mosquito transmission. A review of these productive research fields falls outside the scope of this paper, which will focus on improvements of cultivation and purification techniques in the *P. berghei*/rodent model. The availability of these techniques enhances the value of this particular model in comparison with other malaria models, for studies on the molecular biology of the asexual bloodstages and of sexual development.

The life cycle of *P. berghei* (which has similarities with that of *P. vivax*-like parasites) shows several specific features that facilitate particular areas of research. A distinct characteristic of *P. berghei*, which it shares with *P. vivax*, is the strong preference of the bloodstages for invasion of reticulocytes, which has consequences for *in vitro* cultivation and the purification of the bloodstages (see below). This preference may also influence susceptibility to drugs[12]. Another distinctive property of *P. berghei* is the asynchronicity of bloodstage development in natural infections. The asexual cycle of the haploid bloodstages is short (22–23 h)[14]. Several aspects of the sexual development of *P. berghei* are quite distinct from those of *P. falciparum* and, again, are more similar to *vivax*-type parasites[14]. In *P. berghei* 10–20% of merozoites of each asexual cycle develops into gametocytes. The period of development of gametocytes is short (only 26 h) (similar to the duration of the asexual cycle). The mature, spherical gametocytes remain infective for mosquitoes for a further 26 h. These characteristics of gametocyte development of *P. berghei* allow for the simple collection of large numbers of mature gametocytes (see below). The cycle of *P. berghei* in the anopheline mosquito is similar to that of other mammalian species[5]. Zygotes develop within 18–24 h to mature ookinetes in the mosquito midgut, and these penetrate the midgut wall and transform, within 12–13 days, into mature oocysts. The exo-erythrocytic (EE) development in the liver is short[15]: after invasion of parenchymal cells, sporozoites develop within 43–52 h into mature EE schizonts.

In vitro culture of various developmental stages

In vitro cultivation is often indispensable for collection and purification of different parasite developmental stages for molecular studies. In addition, *in vitro* cultures improve the ease of both the manipulation of the parasites and experimentation, for example the testing of the effect of chemotherapeutic agents and other inhibitors on cell development. A wide range of culture techniques for the various stages of *P. berghei* is available[1].

Culture of bloodstages. Plasmodium berghei is one of the few mammalian parasites whose bloodstages can be cultured *in vitro*[16,17]. Culture conditions (such as culture medium, gas and temperature) closely resemble the conditions for culture of *P. falciparum*, and the growth of young bloodstages into mature schizonts is straightforward (Fig. 1). However, for continuous culture (in which re-invasion of erythrocytes must take place) specific conditions are a prerequisite[16]. Because of the preference of *P. berghei* for reticulocytes, the regular addition of blood with a high percentage of reticulocytes to the cultures is required. The most critical factor in cultivation is the rupture of mature schizonts and the release of merozoites. In static layer cultures, schizonts of *P. berghei* will not burst spontaneously, preventing the merozoites from invading new cells. This characteristic is very useful for the synchronization of bloodstage development (see below), but obviously poses a problem for continuous culture. For maximal re-invasion rates, an artificial mechanical force for the rupture of schizonts is essential; this can be provided by a spinning magnetic bar at the bottom of culture flasks. These two modifications are the only novel features for continuous cultivation of *P. berghei*.

Culture of zygotes, ookinetes and sporogonic stages. Simple and reproducible methods for the large-scale *in vitro* production of zygotes and ookinetes are only available for *P. berghei*[18–20] and *P. gallinaceum*[21]. Fertilization and ookinete development occur readily when (purified) gametocytes are added to standard ookinete culture medium (see Fig. 1). Unlike the avian parasite, *P. gallinaceum*[21], an efficient *in vitro* system for the large-scale production of oocysts of *P. berghei* has not yet been developed. For studies on cell differentiation during oocyst development, one is dependent on the isolation of midguts from heavily infected mosquitoes, and these are readily available[22].

Culture of exo-erythrocytic stages. Reproducible *in vitro* culture systems have been described for high-density EE-stage development of *P. berghei*[23,24]. The highest yield of EE schizonts is obtained by culturing sporozoites in human hepatoma cell lines. Viable merozoites are produced in these cultures, and these are able to invade erythrocytes[25].

Collection and purification of the different developmental stages

Large numbers of the different developmental stages, free from contamination with other developmental stages or host cells, are required for molecular and biochemical investigations. The use of *in vitro* cultures to produce large numbers of bloodstages is hampered in *P. berghei* as a result of its preference for reticulocytes, which precludes mass *in vitro* cultivation[17]. We have adapted a previously developed method for the synchronization of bloodstage development[14] to provide sufficient material of the asexual bloodstages and the sexual stages (see Fig. 1 for details).

Asexual bloodstages and sexual stages. The starting material for the establishment of the synchronized development of bloodstages of *P. berghei* is purified mature schizonts. It is not difficult to obtain large quantities of these cells, free of contamination (Fig. 1a). The schizonts can be used for different purposes. They can be exploited to give preparations of pure viable merozoites (Fig. 1b). *Plasmodium* merozoites have an extremely short survival time (10 min)[26]; merozoites of *P. berghei* survive within the host erythrocyte in culture for 5–8 h and for at least 30 min after being released from their host cell[27] (C.J. Janse and A.P. Waters, unpublished), which allows a less-complicated manipulation of these fragile stages.

The purified schizonts and merozoites provide excellent material for the synchronization of the *in vivo* and *in vitro* development of the bloodstages (Fig. 1c). Intravenous inoculation of schizonts in rats results in the release and re-invasion of merozoites within 3 h (Ref. 14). To collect the various, highly synchronized bloodstages (such as ringforms, trophozoites and young

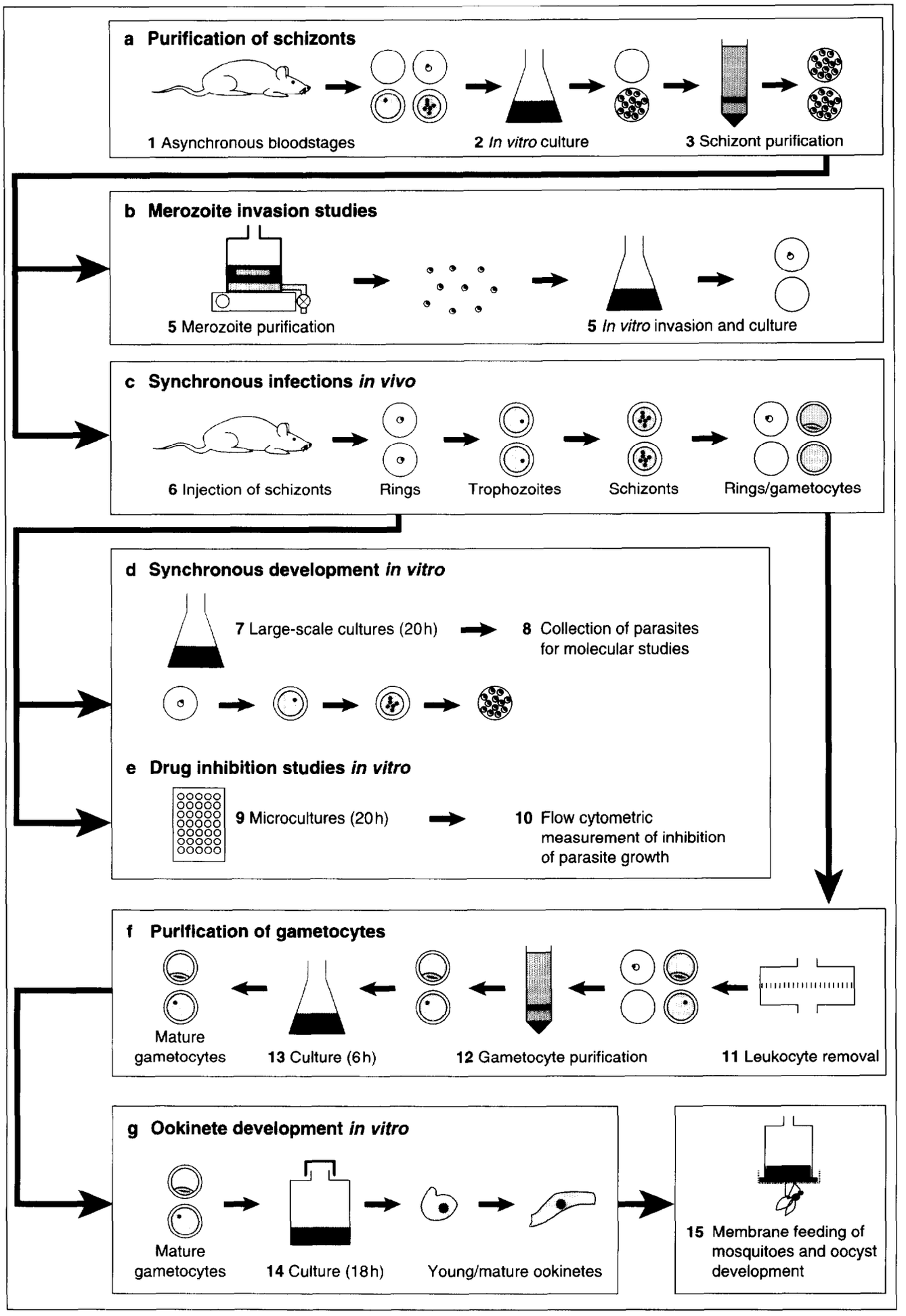
a Purification of schizonts
1 Asynchronous bloodstages
2 In vitro culture
3 Schizont purification
b Merozoite invasion studies
5 Merozoite purification
5 In vitro invasion and culture
c Synchronous infections in vivo
6 Injection of schizonts
Rings
Trophozoites
Schizonts
Rings/gametocytes
d Synchronous development in vitro
7 Large-scale cultures (20h)
8 Collection of parasites for molecular studies
e Drug inhibition studies in vitro
9 Microcultures (20h)
10 Flow cytometric measurement of inhibition of parasite growth
f Purification of gametocytes
Mature gametocytes
13 Culture (6h)
12 Gametocyte purification
11 Leukocyte removal
g Ookinete development in vitro
Mature gametocytes
14 Culture (18h)
Young/mature ookinetes
15 Membrane feeding of mosquitoes and oocyst development

Fig. 1. Collection and purification of synchronized asexual bloodstages and sexual stages of *Plasmodium berghei*. The starting material for the establishment of synchronized development is purified, mature schizonts **(a)**. To collect schizonts, we incubate 30 ml of infected blood (obtained from Wistar rats by cardiac puncture) **(1)** under standard conditions in gently shaken Ehrlenmeyer flasks for 22 h **(2)**. [NB Standard culture conditions are: RPMI1640 medium, pH 7.3; 20% fetal calf serum; 10% O_2, 5% CO_2, 85% N_2 gas mixture; 1–4% cell suspension; 37°C (Ref. 17).] The starting material comprises mainly ringforms and young trophozoites at a parasitemia of 2–3% (first wave of parasitemia after mechanical blood infection). In culture, these parasites grow into schizonts, which do not rupture. Mature schizonts are separated from uninfected cells on density gradients **(3)**. The culture suspension is layered on top of a 55% Nycodenz/PBS gradient and centrifuged for 25 min at 200*g*. The schizonts are collected on the interface, providing preparations consisting of more than 95% pure schizonts ($3–4\times10^9$ schizonts), contaminated with leukocytes and some old gametocytes from the previous cycle. To collect pure viable merozoites **(b)**, the schizonts are ruptured in a simple culture chamber in which a magnetic bar is vigorously spinning on top of a polycarbonate sieve of 1–2 μm pore size **(4)**. The spinbar is spinning directly on the sieve to rupture schizonts by shearing forces. When the culture medium is pumped through the chamber, the free merozoites pass the filter and are collected **(5)** (Ref. 17). For synchronization of the *in vivo* and *in vitro* bloodstage development, purified schizonts are intravenously inoculated in rats which have been treated five days earlier with phenylhydrazine HCl **(6)** (Ref. 14). Inoculation of mature schizonts results in the release and re-invasion of merozoites within a narrow time window of 3 h. Usually, we inoculate approximately 5×10^8 schizonts per rat, resulting in a parasiteamia of 2–3%, which equals an invasion rate of 2–5 merozoites per schizont. We prefer this method of *in vivo* re-invasion following *in vitro* synchronization, since re-invasion of *P. berghei* in rodents, *in vivo*, is more efficient than in culture. Three hours post-inoculation (hpi) of schizonts, the parasite population in the rats consists of more than 95% young ringforms (with residual gametocytes and non-viable schizonts) which develop synchronously either to mature schizonts or to mature gametocytes **(c)**. Infected blood is collected by cardiac puncture at 4 hpi and incubated under standard culture conditions **(7)** after removal of leukocytes, in order to acquire the different synchronized bloodstages **(8)**, such as ringforms, (young) trophozoites and (young) schizonts. To remove leukocytes, blood is passed through a NPBI LF500 leukocyte filter[28] or a Plasmodipur filter[43], both of which are more efficient and practical than columns of CF11-powder. To test the susceptibility of parasites to therapeutic agents and to determine the stage specificity of drugs, synchronized bloodstages are incubated in 24-microwell plates **(9)**, under standard culture conditions, in the presence of different concentrations of the drugs[29,30]. Inhibition of parasite development is determined by measuring (by flow cytometry) the amount of parasite DNA synthesis **(10)**. This technique is a quantitative, rapid, simple and reproducible method to establish drug-susceptibility[44]. To purify gametocytes **(f)**, blood is collected from synchronous infections in rats at 26 hpi. Usually, we collect 30 ml of infected blood from which the leukocytes are removed by filtration through NPBI LF500 filters **(11)**. Synchronous *in vivo* infections contain at 26 hpi mainly ringforms and (immature) gametocytes **(c)** (Ref. 14). The gametocytes are separated from uninfected and ring-infected erythrocytes on density gradients **(12)**. The blood suspension is mixed with eight times the volume of standard culture medium and layered on top of a 48% Nycodenz/culture medium gradient. These gradients are centrifuged for 25 min at 200*g*. Gametocytes collect at the interface of the gradients. All procedures are performed at 37°C to prevent gametocyte activation. Under optimal conditions this procedure provides preparations of about 95% gametocytes, mixed with 5% schizonts with a total of about 5×10^8 gametocytes. For complete maturation, the purified gametocytes are incubated for 6 h under standard culture conditions **(13)**. To obtain high-density synchronous zygote and ookinete cultures **(g)**, the purified, mature gametocytes are incubated in standard ookinete culture medium in small culture flasks at 21°C **(14)** (Ref. 18). (NB the standard culture conditions are: RPMI1640 medium, pH 8.0, without $NaHCO_3$; 10% fetal calf serum; 1% cell suspension.) In these cultures usually 30–90% of the female gametocytes are fertilized and develop into ookinetes, providing preparations of approximately 10^8 ookinetes, principally free from asexual parasites and gametocytes but contaminated with unfertilized gametes. To test the viability of ookinetes, these stages are fed to mosquitoes by membrane feeding **(15)** as described[45].

schizonts), we collect the blood from the rats 4 h after inoculation of schizonts and incubate the newly invaded parasites under standard culture conditions[17] after removal of the leukocytes by filtration[28] (Fig. 1d). The parasites develop in this system with the correct periodicity and synchronicity, and reach full maturity[14].

The synchronous *in vivo* and *in vitro* bloodstage development is a useful tool for determining the stage-specific action of therapeutic agents[29,30] (Fig. 1e). Direct comparison of *in vitro* and *in vivo* drug susceptibility is not possible in other malaria models. In several studies it has been found that the *in vitro* activity of drugs against *P. falciparum* does not correspond to the *in vivo* activity against rodent parasites[30]. It is unknown whether this difference in sensitivity is due to intrinsic differences in susceptibility of the parasite species or to the pharmacokinetic behaviour of the drug in the host. Therefore, the *in vitro* culture of *P. berghei* provides a valuable technique for a comparison of drug susceptibility of *P. falciparum* and *P. berghei*, thereby excluding the influence of host factors. In addition, the effect of drugs can be tested on a wide range of developmental stages of *P. berghei*, since most stages can be cultured reproducibly. This can provide a better understanding of the action and target of drugs.

The collection of pure preparations of large numbers of gametocytes is apparently an elaborate task in malaria research. Usually only a low percentage of the parasite population consists of gametocytes and, furthermore, young gametocytes of many *Plasmodium* species, including *P. berghei*, cannot be distinguished from asexual trophozoites. Mature gametocytes have the same density as old trophozoites and (immature) schizonts, impeding the purification of the mature stages from asynchronous infections by density-gradient centrifugation. Fortunately, in synchronized *in vivo* infections at 26 h after initiation, only two parasite stages are present in the blood of the rats: young rings (initiating the second asexual cycle) and immature gametocytes (from the first cycle) (Fig. 1c). The latter are easily separated from uninfected and ring-infected erythrocytes by density-gradient centrifugation (Fig. 1f). The use of synchronized infections is the only reproducible method for the collection of large numbers of viable gametocytes of *P. berghei*. The purified gametocytes can be used for high-density synchronous zygote and ookinete cultures by incubation of those cells in standard ookinete culture medium[18] (Fig. 1g). The complete synchronization procedure may seem laborious and complicated, but the different techniques, such as cultivation of schizonts, leukocyte removal and density-gradient centrifugation, for the purification of schizonts and gametocytes are simple and reproducible, allowing laboratories with experience with the *in vitro* cultivation of malaria

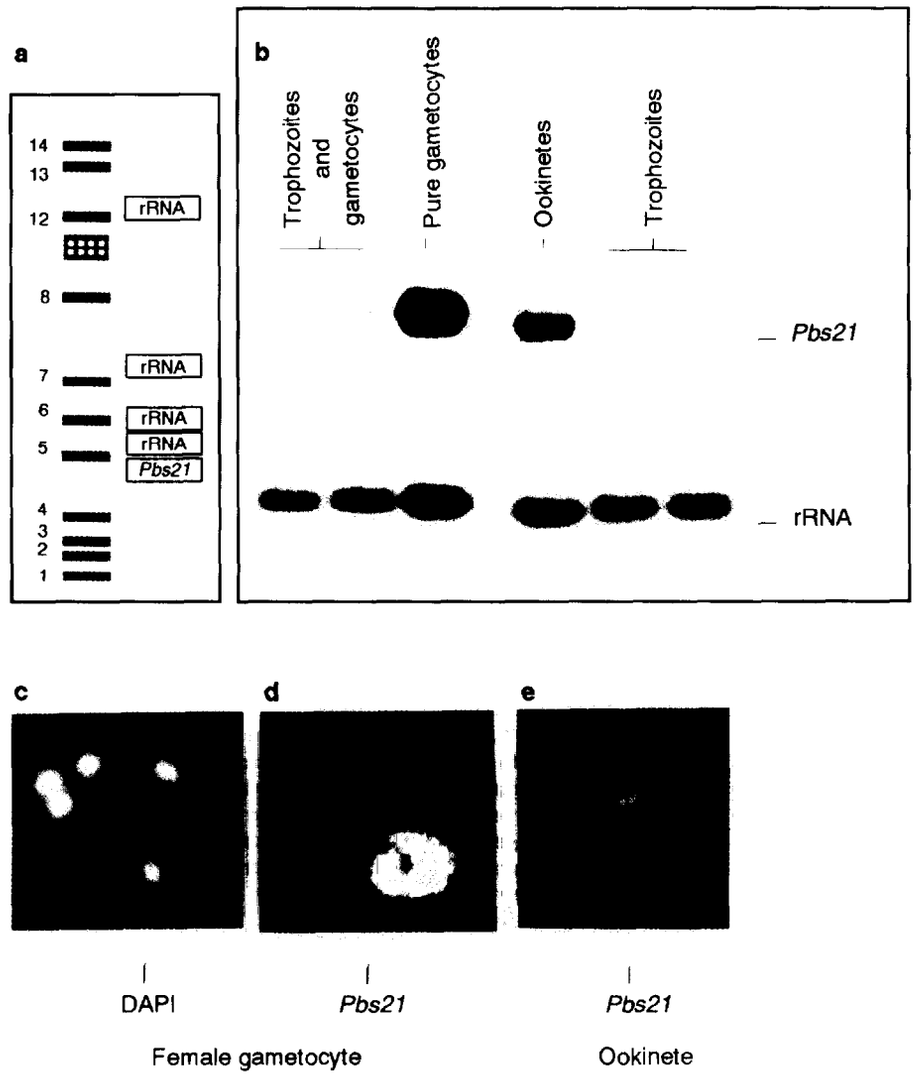

Fig. 2. Differential expression of a gene (*Pbs21*) encoding a surface protein of ookinetes as studied in the *P. berghei*/rodent model. The chromosomal location of *Pbs21* together with the four ribosomal-RNA genes is shown in (a). Expression of this gene is regulated both by transcriptional and post-transcriptional processes[38]. A northern analysis (b) shows the presence of mRNA of *Pbs21* and the rRNA genes in different, purified stages. *Pbs21* is observed in zygotes 1–2 h after fertilization, but mRNA is already abundantly present in mature gametocytes (b, Lane 3) where it is translationally repressed. Transcription of this gene starts in immature gametocytes/trophozoites (b, Lanes 1 and 2) and is absent in trophozoites of non-gametocyte producer 'mutants' of *P. berghei* (b, Lane 3). By fluorescence *in situ* RNA hybridization of *Pbs21* to synchronized bloodstages[39], we found that transcription is restricted to female gametocytes and starts before morphological differentiation of asexual trophozoites and immature gametocytes. Three bloodstages (a multinucleated young schizont, a trophozoite and a female gametocyte) which are stained with the DNA specific dye DAPI (nuclear fluorescence) and simultaneously hybridized to fluorescently labelled *Pbs21* probe (cytoplasmic mRNA fluorescence) are shown (c and d). *Pbs21* mRNA is still detected by *in situ* hybridization in the cytoplasm of mature ookinetes (e)[39,46].

parasites rapidly to introduce the synchronization procedure.

Sporogonic stages and exo-erythrocytic stages. No improvements have been reported recently for the mass collection and purification of sporogonic stages of *P. berghei*. Large numbers of sporozoites are usually collected by density-gradient centrifugation or column purification.[22] Exo-erythrocytic stages can be obtained from *in vitro* cultures of the liver stages. Large-scale, high-density cultures are readily handled by a small team, and produce enough protein for immunization and biochemical studies[24].

Molecular biology of *P. berghei* and the study of cell differentiation

Knowledge of the basic molecular biology of the organism under study and the availability of advanced molecular techniques are required to complete a model for substantial studies on cell differentiation. *Plasmodium falciparum* and *P. berghei* are the most-extensively studied at the basic genetic level of genome organization and mitotic and meiotic processes. The time and rate of DNA replication during mitosis and meiosis have been studied in *P. berghei*[31]. Its 14 chromosomes are well defined by pulsed field gel electrophoretic (PFGE) separation and visualization[32] and the chromosomal location of many genes is known[8]. Several structural elements of the genome, such as telomeres[33] and (subtelomeric) repeat sequences[34], have been characterized. Several genes that are expressed during early sexual development are clustered on a specific chromosome[8], and deletions and rearrangements in this chromosome coincide with the loss of the capacity to produce sexual cells[35] (M. Ponzi, pers. commun.). Further characterization of this chromosome and of the genetic mutants of *P. berghei* (which cannot produce gametocytes[35]) is an interesting area of research that may lead to the elucidation of processes involved in sexual differentiation in *Plasmodium*.

Knowledge of the basic molecular processes involved in cell differentiation of *Plasmodium* (eg. control of gene expression) is limited[36]. The basic structural DNA elements involved in the control of gene expression are not yet defined. This is partly due to the lack of functional molecular assays, such as genetic transformation and transcription assays, which are essential for these studies. In *P. berghei* we use two model genes to study the regulation of differential gene expression during asexual and sexual development. The first is *Pbs21*, a gene encoding a surface antigen of ookinetes[37]. By a combination of synchronized development of the sexual stages, northern blot analysis and RNA *in situ* hybridization using different parasite mutants, it has been shown that expression is sex specific and is regulated

both by transcriptional and by post-transcriptional control[38,39] (Fig. 2). With the availability of large numbers of the sexual stages of *P. berghei*, and the application of transcript mapping and newly developed transient transfection of zygotes[40], the functional study of both control mechanisms can now be addressed. The second class of genes for the study of the control of differential gene expression in *P. berghei* is the developmentally regulated ribosomal RNA genes, which were first described in this species[41]. *Plasmodium berghei* has only four copies of these genes (A–D), of which three are now extensively characterized with regard to stage-specific expression and the processing of the RNA transcripts[42] (A.P. Waters, unpublished). Given the relative genetic simplicity of the *P. berghei* rRNA genes, combined with the sophistication of culture and purification of the different developmental stages, this class of gene is an ideal model in which to study the unique phenomenon of developmentally regulated ribosomes. The rRNA genes also offer an advantage in the development of new genetic systems in *Plasmodium* for the study of gene expression. Their low copy number indicates that the rRNA gene promoters must be extremely powerful, and therefore ideal test candidates to drive the expression of reporter elements in both *in vitro* and *in vivo* transcription assays. This subject area is more comprehensively reviewed and redefined in the accompanying article by McCutchan *et al.* (this issue).

Conclusion

A major challenge in future research on the control of gene expression and cell differentiation in *Plasmodium* is the development of the above-mentioned, advanced molecular techniques for malaria research. We believe that the *P. berghei*/rodent model can make a significant contribution to the development and application of these techniques, which are indispensable for studies on the fascinating cyclic differentiation of *Plasmodium*, and may contribute to the discovery of new targets for chemotherapeutic agents.

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