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Cultivation of *Plasmodium* spp.

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INTRODUCTION

On a global scale, malaria has been and remains a major public health concern (54). The disease is caused by parasitic protozoa of the genus *Plasmodium*. The life cycle of this organism is complex, with the parasite alternating between sexual reproduction in an invertebrate (mosquito) host and asexual reproduction in a vertebrate host (80). In addition to mammals as vertebrate hosts, birds and reptiles also serve as hosts for malarial parasites. The portion of the life cycle in the mosquito is the sporogonic phase, leading to formation of sporozoites which are injected by the vector into the vertebrate host at time of feeding. Sporozoites give rise to the schizogonic phase, with proliferation of the parasites in erythrocytic and exoerythrocytic sites. The parasite is extracellular during its sporogonic phase, shifting to an intracellular location during the schizogonic stages of development. In vitro cultivation of the parasite requires simulating conditions in the mosquito vector for the sporogonic phase of the life cycle and, for the schizogonic phase, conditions promoting growth in exoerythrocytic and erythrocytic locations of the vertebrate hosts. In this review, we attempt to bridge some of the earlier literature to recent developments relating to in vitro growth of *Plasmodium* spp. Both human and non-human malarial parasites are dealt with in this section, even though the latter are not of clinical significance. Non-human *Plasmodium* spp. share nutritional characteristics with their human counterparts, and have often served as models for cultivation of species infecting humans. Since this review does not deal in detail with the extensive literature on cultivation of *Plasmodium* spp., the interested reader is referred to the more comprehensive treatments of cultivation of the various stages in the parasite life cycle (see, for example, references 6, 39, and 93 for reviews of the exoerythrocytic, erythrocytic, and sporogonic stages, respectively).

When introduced into the bloodstream of a vertebrate host by a mosquito, sporozoites enter into the exoerythrocytic phase of development. In mammals, this occurs in hepatic cells, while in avian hosts, the parasites invade cells of the reticuloendothelial system (80). In mammalian malarias, the invasive stage

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for erythrocytes, the merozoite, passes through a developmental sequence beginning with a characteristic ring stage and leading to formation of a multinucleate schizont. Further development of the schizont leads to formation of multiple merozoites, which upon rupture of the infected host cell, invade other erythrocytes. In avian malarias, after exiting from cells of the reticuloendothelial system, the merozoites can either reinvade reticuloendothelial cells or enter erythrocytes. Parasites can be recovered from the peripheral bloodstream of the vertebrate host, where they go through a cycle of repeated invasion of erythrocytes with characteristic synchrony to increase the level of parasitemia in the host bloodstream. Humans are infected by four species of malaria parasites: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. To a greater or lesser extent, all four species have been cultured or maintained in vitro; *P. falciparum*, however, is the only species for which all life cycle stages have been established in culture (27).

Differences exist between strains of *Plasmodium*. Some strains are readily established in vitro, while others are refractory to cultivation. Isolates undergo change once in culture, perhaps due to selection. In *P. falciparum*, for example, gametocyte formation is typical of recently cultured strains but is lost with prolonged cultivation. In this regard, it is important to note that cryopreservation of isolates can maintain those characteristics in a strain that may be lost on prolonged cultivation (68). Also, the use of cloned cell lines for experiments and the characterization of laboratory strains based on proteins and DNA is another consideration (70).

HUMAN MALARIAS

Exoerythrocytic Stages

When the parasite is first introduced into the bloodstream of its vertebrate host by the mosquito vector, the sporozoite stage of *P. falciparum* invades hepatic cells. Aspects of exoerythrocytic cultivation have been reviewed by Hollingdale (26), Jensen (38), and Trager and Jensen (87).

In the case of *P. falciparum*, the parasite is probably taken up first by the Kupffer cells of the liver sinusoids in their passage to liver hepatocytes (33). Mazier et al. (52) have successfully infected hepatocytes prepared from human liver biopsy specimens in minimal essential medium (MEM) with sporozoites of *P. falciparum*, as determined by indirect immunofluorescence staining. Invading sporozoites developed into schizonts in host cells, with modest yields of about 650 schizonts/35-mm-diameter culture dish. Addition of human erythrocytes to infected hepatocyte cultures resulted in appearance of ring-stage organisms in the blood cells, indicating production of merozoites infective for erythrocytes. Calvo-Calle et al. (11) observed that *P. falciparum* developed within human hepatoma cell lines huH-1 and huH-2 but not consistently (one out of three experiments). A different human hepatoma cell line (HHS-102) allowed development of liver stages of *P. falciparum* and formation of ring stages in cocultured erythrocytes (43).

The pattern of relapse that occurs with the human malarias caused by *P. vivax* and *P. ovale* is associated with the presence of dormant parasites termed hypnozoites that survive in the liver parenchymal cells of the host. Hollingdale et al. (28) compared dividing and nondividing stages of *P. vivax* in hepatoma cells (HepG2-A16). They found that ca. 1 in 10^4 sporozoites was infective for hepatoma cells, with merozoite release occurring on day 9 in vitro, compared to release on day 5 to 6 in vivo. They observed a population of smaller, nondividing parasites in infected hepatoma cells, which they believed to be hypnozoites. Primary human hepatocytes successfully supported transformation and maturation of exoerythrocytic stages of *P. ovale*, while other cell types (HepG2 and rat hepatocytes) would support transformation only (53). Developmental liver stages of *P. malariae* were produced in primary hepatocytes from the chimpanzee (56).

Erythrocytic Stages

The greatest amounts of effort and time have been invested in cultivation of the erythrocytic stages in the *Plasmodium* life cycle, this being the stage most often associated with the pathogenesis of malaria and a major target for vaccine development. A significant accomplishment in this area was defining in vitro conditions for continuous cultivation of *P. falciparum*, the most important and deadly of the human malarial parasites. This was accomplished by Trager and Jensen (85, 86) using HEPES-buffered RPMI 1640, a tissue culture medium developed for in vitro cultivation of leukocytes, supplemented with human serum, erythrocytes, and sodium bicarbonate. Parasites were cultured initially in petri plates placed in a candle jar that provided an atmosphere of 3% CO_2–17% O_2 or in vials that allowed for continuous flow of medium into culture vessels with an atmosphere of 7% CO_2–1% O_2–92% N_2. Later efforts gave rise to various continuous-flow devices (37, 67, 78, 81, 86), as well as suspension cultures (68, 100) for improved control and yields.

Serum as medium supplement. The growth system functions best with 10 to 15% human serum as a supplement (85). For reasons that include cost, reproducibility, and possible presence of inhibitory immune factors and antimalarial drugs, there is interest in substituting other types of mammalian sera (bovine, monkey, horse, goat, sheep, rabbit, or swine) for human serum or even developing a serum-free medium for parasite cultivation. In comparing horse, swine, and lamb sera, horse serum was superior to the others but not as good as human serum (10). Fetal bovine serum, generally less effective for growth than human serum (86), when freshly obtained from fetuses of different breeds of cattle produced good parasite growth initially but led to a decline in numbers of parasites over 30 days (37). Rabbit serum (5 to 10%) was used in place of human serum but required a 2- to 3-week period of adaptation of cultures (73). Jensen (38) compared percentages of growth of *P. falciparum* in sera of different animal origins. With fresh human serum as a standard at 100%, the following sera were rated as indicated: fresh fetal bovine, 35%; adult bovine, 7%; newborn bovine, 1%; horse, 19%; swine, 14%; and sheep, 2%. Ifediba and Vanderberg (32) reported replacement of human serum by neopeptone and Proteose Peptone no. 3; bovine serum albumin (5 g/liter) was reported to replace serum by Ofulla et al. (65). Dialyzed human serum lost its ability to support growth of parasites, and commercial samples of human serum supported growth at about one-quarter that of control cultures (37). Heat-inactivated, semi-immune human plasma from a region of malaria endemicity was used success-

fully for continuous cultivation of primary isolates of *P. falciparum* (64).

Serum replacements. Freshly prepared human high-density lipoprotein fraction (concentration range of 0.25 to 0.50 mg/ml) was used to support growth of *P. falciparum*, with results comparable to those obtained using human serum (23). Other lipoprotein fractions, low- and very-low-density lipoproteins, produced little or no growth. Growth-promoting factor GF 21 (containing an ammonium sulfate fraction of adult bovine serum plus insulin, transferrin, and sodium selenite) was used with Daigo's T basal medium for serum-free growth of *P. falciparum* (2). RPMI 1640 was supplemented with adenosine, unsaturated C_{18} fatty acids, and fatty acid-free bovine serum albumin for serum-free growth, but growth rates of parasites were lower than those in plasma-containing medium (98). Pooling sera minimized variations in growth-promoting properties of serum samples obtained from different humans (39) and rabbits (73).

Commercial serum replacements. Lingnau et al. (51) used a commercially available serum replacement preparation, Nutridoma-SR (4%), to support the growth of several strains of *P. falciparum* from different global locations, with a resulting parasitemia of about 10% within 3 to 4 days. Flores et al. (18) had better results using a lower concentration of Nutridoma-SR (1%) combined with Albumax I (0.5%), a purified serum albumin preparation. Cultures were maintained for 30 to 50 days, with parasitemias of 10%, compared to parasitemias of >15% obtained with human serum. They found that cultures raised in higher concentrations of Nutridoma-SR (2 or 4%) were nonviable or gave lower levels of parasitemia (parasitemia being the level of infection of blood cells). Binh et al. (8) also used Albumax for cultivation of *P. falciparum*, with parasitemias reaching as high as 85% after 7 days with continuously passaged plasmodia. Cranmer et al. (15), using Albumax II (0.5%) for growth of *P. falciparum*, achieved parasitemias of about 6 and 12% for two different malaria strains. They found that it was necessary to add hypoxanthine to the growth medium in order to obtain these levels of parasitemia. Plasma, without prior heat treatment, has been used for large-scale growth of *P. falciparum* (69); clotting was avoided by use of plastic culture vessels or siliconized glassware.

Basal medium. The tissue culture medium RPMI 1640 (Table 1) remains the medium of choice, not only for *P. falciparum* but also for most other *Plasmodium* spp. that have been cultured in vitro (see below). Better consistency for parasite growth is obtained by preparing the medium from a powdered preparation rather than using the liquid form available from most suppliers (39). The medium is supplemented with hypoxanthine as a purine source (82). RPMI 1640 has been supplemented with additional glucose, hypoxanthine, and reduced glutathione (100) to improve parasite yield. Divo et al. (17) produced a semidefined growth medium containing hypoxanthine as the preferred purine source; calcium pantothenate; and the amino acids cystine, glutamate, glutamine, isoleucine, methionine, proline, and tyrosine. Glucose could not be replaced by other sugars: ribose, mannose, fructose, galactose, and maltose (20). Antimetabolites of riboflavin, nicotinamide, pyridoxine, and thiamine were inhibitory to growth, expressed as incorporation of [^{3}H]hypoxanthine, in semidefined medium (21).

TABLE 1. Composition of RPMI 1640 tissue culture medium[a]

Component	Amt (mg/liter)
Inorganic salts	
$Ca(NO_3)_2 \cdot 4H_2O$	100.0
KCl	400.0
$MgSO_4$ (anhydrous)	48.8
NaCl	5,300.0
$NaHCO_3$	2,000.0
Na_2HPO_4 (anhydrous)	800.0
Amino acids	
L-Arginine	200.0
L-Asparagine (free base)	50.0
L-Aspartic acid	20.0
L-Cystine · 2HCl	65.0
L-Glutamic acid	20.0
L-Glutamine	300.0
Glycine	10.0
L-Hisitidine (free base)	15.0
L-Hydroxyproline	20.0
L-Isoleucine	50.0
L-Leucine	50.0
L-Lysine · HCl	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline	20.0
L-Serine	30.0
L-Threonine	20.0
L-Tryptophan	5.0
L-Tyrosine · 2Na · $2H_2O$	29.0
L-Valine	20.0
Vitamins	
D-Biotin	0.20
D-Ca pantothenate	0.25
Choline chloride	3.0
Folic acid	1.0
I-Inositol	35.0
Niacinamide	1.0
para-Aminobenzoic acid	1.0
Pyridoxine HCl	1.0
Riboflavin	0.20
Thiamine HCl	1.0
B_{12}	0.005
Other components	
D-Glucose	2,000.0
Glutathione (reduced)	1.0
HEPES	5,958.0
Phenol red	5.0

[a] From the GIBCO BRL product catalog. Formula for 25 mM HEPES-buffered liquid RPMI 1640 at 1× concentration, with L-glutamine. Components and concentrations may vary with the state of the medium: 1× or 10×, liquid or powder, with or without glutamine, or with or without HEPES buffering.

Role of the erythrocyte in the culture system. Red blood cells are essential for development of the parasite, providing not only a location for asexual reproduction but also a source of nutrients for the parasite over and above that present in supplemented RPMI 1640. In vivo or in vitro, the parasite enters into the erythrocyte across the membrane and is enclosed in a vacuole, the parasitophorous vacuole, that forms in part from the membrane of the red blood cell. The malaria parasite takes in nutrients and develops into a multinucleate schizont which undergoes fission to produce a characteristic number of merozoites which, upon rupture of the parasitized

blood cell, invade other blood cells and repeat the growth cycle. Human blood cells of all groups are suitable for growth of *P. falciparum* (11, 39). Type O cells are useful because of their compatibility with serum or plasma of all other blood groups (69). Trager (82) has used a combination of type A cells and serum because of availability. Type AB serum is compatible with any type of red blood cells. Citrated red blood cells may be stored for up to 5 weeks, at which time they become too fragile to use in cultures (86). Citrated red blood cells are washed and prepared as a 50% erythrocyte suspension which remains usable for 4 days at 4°C. Saline-adenine-glucose-stored blood cells were found to improve parasite yield (100). Chimpanzee, but not rhesus monkey or guinea pig, red blood cells supported development of *P. falciparum* (25).

Culture systems. Plate cultures are prepared with a 5% hematocrit and about 1% parasitemia. The lower the initial parasitemia is, the greater the increase in numbers of parasites that will occur during in vitro growth is. Trager (82) obtained 20- to 50-fold increase in parasite numbers with a starting parasitemia of 0.1%. Parasitemia of cultures can be increased to about 20% by changing medium in cultures every 8 h (41). Monitoring of parasitemia is accomplished by preparing blood films, staining with Giemsa stain following methanol fixation, and counting infected red blood cells microscopically.

While the simplest system for cultivation of parasites uses petri or Linbro plates in a candle jar, this system is labor-intensive, requiring constant attention and daily changes of medium in order to maintain parasite growth. In this static system infected erythrocytes settle out to form a layer, producing microenvironments high in lactic acid in the region of the proliferating parasites. This may lead to conditions unfavorable for schizont development and penetration of merozoites into uninfected erythrocytes. Lactic acid production taxes the buffering capacity of the medium and leads to a drop in pH, which is detrimental to the growth of *Plasmodium*. Optimal parasite yields occurred with an extracellular pH of 7.2 to 7.45 and a lactate concentration below 12 mM (42); higher lactate concentrations were postulated to cause negative feedback of glycolysis. Glucose diffusion into the cell layer also becomes limiting (41).

Several devices to semiautomate cultivation of plasmodia have been described previously (38, 39, 68, 79, 82). These devices allow for continuous flow of medium through growth vessels, with a controlled gas phase of 2 to 5% CO_2, 3 to 18% O_2, and the remainder being N_2. Semiautomated devices reduce the amount of time spent on maintenance of stock cultures of the parasite. Although the malarial parasite is found in red blood cells, it is microaerophilic in its oxygen preference (74). Taylor-Robinson (78) used a commercial system designed for growth of *Campylobacter* spp. to generate the low O_2 and high CO_2 levels favored by *P. falciparum*. Anaerobic jars and gas-generating envelopes were employed to grow parasites in tissue culture flasks or microtiter plates in an atmosphere of 6%O_2–8% CO_2–86% N_2 (78). Suspension cultures of parasites have also been tried. Zolg et al. (100) reported improved yields with shaking of cultures, but these claims have been disputed (39). Fragility of the infected red blood cells is an important factor in agitated cultures, but this has been countered to some extent by the use of methylcellulose in agitated cultures (47, 100). Mons et al. (60) have used a culture flask with a stirring bar for cultivation of the rodent parasite *P. berghei* (see below).

Induction of synchrony in vitro. In its human host, *P. falciparum* exhibits a synchrony of about 48-h duration. Blood sampled at any one time from an infected host will reveal a parasite population at the same stage in its developmental cycle, i.e., mostly ring stages or mostly schizonts, etc. This synchrony is in part a response to the circadian rhythmicity of the host's body. Synchrony can be artificially imposed in vitro upon developing malaria parasites by one of several methods. Most popular is the use of sorbitol or mannitol treatment of infected erythrocytes (45). Infected cells are treated with 5% sorbitol, which causes lysis of erythrocytes containing late stages and preferentially selects for red blood cells with early ring stages. The effect is not osmotic, but has to do with the permeability of infected cells and the sensitivity of the parasites to sorbitol (82). Treatment can be repeated at 34 h to further select for young stages and improve on the synchrony. Other techniques involve the separation of late-stage parasites, as by sedimentation in Plasmagel (67) or gelatin (35). Most strains of *P. falciparum* produce knobs over the surface of the host erythrocyte when the parasite reaches the late trophozoite to schizont stage. Such red cells do not form rouleaux in the Plasmagel, as do uninfected red cells or those containing ring-stage organisms. As a result, the cells with late-stage parasites remain in suspension while the uninfected ones and those with rings settle out. If the late-stage parasites are then mixed with fresh red cells and put under culture conditions, merozoites formed by them invade new cells. If one allows invasion to go on for 3 h and then treats the cells with sorbitol to kill all late-stage parasites, one gets a population of rings just 0 to 3 h old. Such a tightly synchronized culture will remain quite synchronous through about three cycles. Shifting cultures of *P. falciparum* from 37 to 28°C has been used to delay the asexual cycle for 12 to 16 h (72).

Awad-El-Kariem et al. (3) have used feeder cells, either mouse peritoneal macrophages or the flagellated protozoon *Crithidia fasciculata*, for establishment of *P. falciparum* in vitro with a success rate better than 80%. While the role of *Crithidia* in facilitating cultivation of malaria parasites is not clear, the authors suggest that it might involve lowering the redox potential of the culture medium, thereby protecting parasites from damage due to reactive oxygen intermediates.

It is worth noting that cultured *P. falciparum* retains its infectivity and immunogenicity. In a laboratory accident, a puncture wound resulted in inoculation of cultured parasites leading to human infection with a strain that had been maintained in vitro for approximately 4 years (40). Trager (81) noted instances in which cultured material was used for vaccine development, indicating retention of immunogenicity.

***Plasmodium* spp. other than *P. falciparum*.** Although the in vitro system has worked well for *P. falciparum*, *P. vivax* has not been amenable to cultivation using the same methods. Brockelman et al. (9) used SCMI 612 medium for schizogonic stages of *P. vivax* and found that this medium worked better than either RPMI 1640 or Weymouth media. They reported that a higher glucose level (3 mg/ml) was needed for *P. vivax* than for *P. falciparum*. Unlike *P. falciparum*, which develops in erythrocytes, *P. vivax* invades developing erythrocytes, the reticulocytes. Thus, in order to maintain this species in vitro, a large

supply of reticulocytes is needed for development. Mons et al. (58, 59) used RPMI 1640 medium with an enriched reticulocyte fraction from owl monkeys (*Aotus* sp.), noting that *P. vivax* preferentially infects these immature erythrocytes. However, levels of parasitemia were low (59). Not only were reticulocytes necessary for parasite development, but agitation of the culture medium was needed to establish contact between parasite and host cell (58). Agitation of cultures led to decrease in the numbers of parasites due to fragility of the parasitized *Aotus* blood cells. A variety of supplements including hypoxanthine, ascorbic acid, choline, biotin, B_{12}, and $MgCl_2$ had little or no effect on growth and schizogony (59). The need for young erythrocytes and reticulocytes was noted by Lanners (47), who used a continuous-flow system. Methylcellulose (0.1%) was added to reduce breakage of erythrocyte membranes. Other supplements included glucose, spermidine, and antioxidants.

Golenda et al. (22) achieved stable in vitro cultivation of *P. vivax* for eight growth cycles by use of enriched populations of reticulocytes from humans with hemochromatosis. Using McCoy's 5A medium supplemented with glutamine and 20% human serum, they cultured the parasite initially in a candle jar until schizogonic stages were produced. The reticulocytes were then added and the culture was transferred to a shaker, which promoted contact between parasites and host cells. They obtained 85% ring-stage organisms after about 12 h under these conditions. Gametocytes did not develop, although the parasites remained infective for owl monkeys.

Lingnau et al. (48) observed development over a 6-day period of erythrocytic stages of *P. malariae* in RPMI 1640 medium supplemented with glutamine, hypoxanthine, and 20% human serum. The variety of stages seen in red blood cells suggested that merozoites were produced in vitro and invaded noninfected erythrocytes.

Cell-Free Development of Erythrocytic Stages

While in vitro growth of plasmodia is a significant accomplishment in its own right, growth of the parasites under a completely cell-free or axenic condition would allow examination of the parasites' nutritional needs, biochemical and molecular properties, and sensitivity to antimicrobial agents, in the absence of those of the host erythrocyte. Towards this end, Trager and coworkers were successful in obtaining merozoite development of *P. falciparum* to ring stages under cell-free conditions. In an early study, Trager and Lanners (89) cultured *P. falciparum* merozoites to ring and trophic stages in HEPES-buffered RPMI 1640 in which NaCl was replaced by KCl, in the presence of a serum-supplemented, frozen-thawed 33% erythrocyte extract; dipotassium ATP (1.6 mM); and pyruvate (3.6 mM). Ultrastructural evidence indicated absence of the parasitophorous membrane in these extracellular forms, yet development occurred, suggesting that factors present in the erythrocyte but not necessarily the intracytoplasmic location of the parasite were needed for development. In a later study, it was found that sonicated erythrocyte preparations (about 50% erythrocyte extract), with ATP (2 mM) and pyruvate (5 mM) added, supported better development of extracellular *P. falciparum* than did frozen-thawed preparations, with about 30% of the merozoites developing to later stages (92). These extracellular forms react to the same monoclonal antibodies that the intracellular forms respond to, suggesting a similar pattern of molecular differentiation (101).

Biphasic cultivation system. Further refinement of the technique for axenic cultivation involved the use of a biphasic system with merozoites embedded in a Matrigel substrate in a well containing a fluid medium overlay (90, 91). Coenzyme A, added as a supplement to the basic medium (0.15 mM), may enhance formation of later stages in the life cycle, though its role was not clear from an earlier study (92). The fluid medium could be changed with minimal disturbance to the parasites developing within the Matrigel layer. Ring-stage parasites developing within Matrigel attained larger sizes and showed motility, though only about 1% of the merozoites inoculated completed the asexual cycle in vitro. Gametocytes were observed in cultures after 36 to 44 h of incubation (90). Trager et al. (90) postulated that contact between parasite and spectrin in the erythrocyte sonicate may be an important factor for development. More recently, Williams et al. (99) have increased the yields of schizonts forming extracellularly by addition of erythrocyte ghosts obtained by osmotic lysis, to double the amount of membrane present. These results support the critical role of the erythrocyte membrane in the development of the malarial parasite. The role of Matrigel, a preparation of solubilized tissue basement membrane, may be simply to provide the parasite with a substrate that approximates the cytoplasmic matrix in which the merozoites begin to develop. Matrigel has also been an important factor in cultivation of the mosquito stages of the *P. falciparum* life cycle (see below).

Gametocytogenesis

Gametocytes are the precursors of the macrogamete (female gamete) and the sperm cell which fuse to form a motile, banana-shaped zygote, the ookinete, in the wall of the gut of the mosquito. In vivo, gametocyte development occurs within erythrocytes in the peripheral circulatory system of the vertebrate host. These stages are picked up by the blood-sucking mosquito, in which they complete the sexual cycle of the malaria parasite. In vitro, variation exists among different strains of *P. falciparum* with respect to gametocyte formation, and even among different clones from the same strain of parasite. As an example of the latter, Bhasin and Trager (7) isolated three clones of a Honduran strain of *P. falciparum*, of which two developed gametocytes and one did not.

Induction of gamete formation. Gametocyte formation in cultures can be enhanced by changing the growth medium without providing fresh erythrocytes (33). Culture conditions also affect gametocytogenesis of *P. falciparum* (44). Strains recently isolated are more likely to form gametocytes than are strains that have been in culture over long periods of time (1, 12). Ifediba and Vanderberg (33) reported that hypoxanthine (50 μg/ml) was necessary for induction and maturation of gametocytes forming in cultures of *P. falciparum*; without hypoxanthine, mosquitoes feeding on gametocyte-containing cultures would not develop oocyst infections. Others have reported that some ammonium compounds (ammonium carbonate or ammonium bicarbonate, but not ammonium chloride or ammonium acetate) with or without concanavalin A trigger gametocyte formation on the third day following treatment (66). Evidence that signal transduction is involved in

TABLE 2. In vitro cultivation of stages of human *Plasmodium* spp.[a]

Stage	Species	Basal medium(a)	Additional factors	Reference(s)
Exoerythrocytic	*P. falciparum*	MEM	Hepatoma cells	11
		RPMI 1640-MEM		43
	P. malariae	MEM	Chimpanzee primary hepatocytes	56
	P. ovale	Arginine-free Ham's medium	Human and rat hepatocytes	53
	P. vivax	MEM	Hepatoma cells	28
Erythrocytic	*P. falciparum*	RPMI 1640		84, 85
		Semidefined medium		17
		RPMI 1640	Cell-free system with Matrigel	89, 98
		Malaria culture medium	Feeder cells (*C. fasciculata*)	3
	P. malariae	RPMI 1640	Glutamine, hypoxanthine	48
	P. vivax	SCMI 612	Elevated glucose	9
		RPMI 1640	Reticulocytes	58, 59
		McCoy's 5A	Reticulocytes	22
Gametocyte	*P. falciparum*	RPMI 1640	Hypoxanthine	33
		RPMI 1640	Hormonal stimulation (insulin, etc.)	49, 50
Sporogonic	*P. falciparum*	RPMI 1640	*Drosophila* cells and Matrigel	94

[a] Summary of the different media used for cultivation of the several species of the malaria parasite and their different stages.

gametocytogenesis comes from several studies in which secondary messengers, or compounds affecting them, have been shown to enhance gametocyte formation. Cyclic AMP (cAMP) and dibutyryl cAMP (1 mM) increased gamete formation in *P. falciparum* (44). Trager and Gill (83) employed phorbol compounds (phorbol 12-myristate-13-acetate and phorbol dibutyrate) and a phosphodiesterase inhibitor (8-bromo cAMP) to promote gametocyte production by 50% or more in cultured *P. falciparum*; they found, however, that cAMP and forskolin were without effect on gametocyte differentiation. Lingnau et al. (49, 50) have examined the role of several hormones in *P. falciparum* gametocytogenesis in serum-free medium. Exflagellation, the differentiation and development of male gametes from a male gametocyte, was accomplished in vitro by Carter and Beach (12) by washing gametocytes in fetal bovine or human serum at pH 8. No evidence of gametocyte formation was seen in the short-term cultivation of *P. malariae* (48).

Problems with induction. Unlike the asexual division cycle that occurs within erythrocytes over a 48-h period, maturation of gametocytes of *P. falciparum* requires about 2 weeks. Nutrients present within the infected erythrocyte would be exhausted by the developing parasite over this prolonged time period (33, 36) and may be a factor in the difficulty of inducing gametocyte formation in vitro. Immature erythrocytes (reticulocytes) supported better gametocyte formation than did mature erythrocytes (84). Other factors affecting gametocyte formation may include variations in nutrients present in serum supplements of growth medium, absence of essential activating factors in RPMI 1640, and selection of non-gametocyte-forming populations under in vitro conditions. Trager (82) concluded that none of the techniques currently available for inducing gametocytogenesis is of practical value, largely due to the difficulties in obtaining gametocytes in quantity. Reviews of gametocytogenesis are available (1, 93).

Sporogonic Stages

The mosquito acquires gametocytes from the blood of a vertebrate. Development of the gametocytes into male and female gametes is initiated within the gut of the mosquito. The fused gametes form an ookinete, which develops into the oocyst in the hemocoel of the insect. Sporozoites develop within the oocyst and migrate to the salivary glands of the mosquito, to be injected into the next vertebrate host that the insect bites. A different set of growth conditions is needed for the successful cultivation of these stages of the *P. falciparum* life cycle. Warburg and Schneider (95) successfully produced sporozoites of *P. falciparum* in a complex culture of *Drosophila melanogaster* cells and a Matrigel substrate, to which ookinetes adhered. Development of sporozoites took 12 to 16 days. Wheat germ agglutinin was used to enhance transformation of zygotes into retorts and ookinetes, by providing a modifying environment approximating conditions in the mosquito stomach for development of the sexual stages. Table 2 presents a summary of the stages of human malarial parasites and the types of media and supplements that have been used in the cultivation.

NON-HUMAN MALARIAS

Malarial parasites develop in the blood of many different vertebrates. In this section, attention will focus on in vitro-cultured malarial parasites infecting simian, avian, and rodent

hosts. In most instances, it is more convenient working with malarial parasites from nonhuman hosts because they can be maintained in vivo, thereby allowing testing for infectivity of in vitro-cultured stages in the vertebrate host and providing an animal model for the study of the parasite in the human host.

Exoerythrocytic Stages

Techniques for cultivation of the exoerythrocytic stages of avian malarial parasites *P. gallinaceum*, *P. lophurae*, and *P. fallax* were described by Huff (31). A variety of tissue culture media supplemented with chicken serum (10 to 20%) and whole egg ultrafiltrate (5%) were used to establish primary cultures of infected chicken embryo cells that could be kept for up to 7 months. In a later study by Davis et al. (16), *P. fallax* was maintained in turkey embryos by successive transfer of infected tissue onto the chorioallanotic membrane of ~2-week-old embryos. Once parasites were established in the embryos, infected brain tissue was removed, trypsinized to obtain a cell suspension, and transferred to flasks containing supplemented medium 199. These cultures could be maintained for two or three passages until heavy parasitism required addition of uninfected embryonic turkey brain cells or transfer of culture supernatant containing merozoites to uninfected normal turkey brain in culture.

Primary cultures of hepatocytes from rhesus monkeys (*Macaca mulatta*) were used to support growth of several simian malarias (*P. cynomolgi*, *P. knowlesi*, *P. coatneyi*, and *P. inui*) by Millet et al. (57). Using sporozoites released from mosquito salivary glands, they found that less than 1% of the sporozoites survived to invade hepatocytes. Fungal contamination was a problem with cultured stages, requiring the addition of flucytosine to the culture medium. An arginine-deficient cell culture medium was used in some instances to prevent overgrowth of hepatocytes before parasite development could be observed in vitro. In a later study, Millet et al. (55) used rhesus hepatocytes for cultivation of developmental stages of *P. fieldi* and *P. simiovale*, two parasites that infect macaques. They found that about 1 in 10^4 sporozoites underwent development into schizonts in the hepatocytes.

The rodent malarial parasite *P. berghei* from in vivo-infected livers was maintained in primary cultures of rodent liver cells by Foley et al. (19). These exoerythrocytic plasmodial stages retained their infectivity for rats. Human embryonic lung cells (W138) and human hepatoma cells (HepG2-A16) were used to cultivate the exoerythrocytic stage of *P. berghei*, with about 8% of the sporozoites developing within the hepatoma cells (29, 30). Two human hepatoma cell lines (huH-1 and huH-2) were used for exoerythrocytic development of *P. berghei* and *P. yoelii* (11). *P. berghei* would also develop in HeLa cells, but *P. yoelii* would not.

Erythrocytic Stages

Erythrocytic development of a number of simian malarial parasites has been studied, including that of *P. knowlesi*, P. *cynomolgi*, *P. fragile*, *P. gonderi*, *P. coatneyi*, *P. inui*, P. *fieldi*, and *P. simiovale*. Some of these aforementioned malarias are nonhuman facsimiles of human malarias. Thus, *P. fragile* and *P. coatneyi* produce falciparum-like malaria in monkeys, *P. cynomolgi* produces vivax-like malaria, *P. inui* produces *P. malariae*-type malaria, and *P. fieldi* and *P. simiovale* produce *P. ovale*-type malaria. With few exceptions, these organisms have been maintained in vitro in their erythrocytic stages.

Simian malarias. *P. fragile* was cultured using RPMI 1640 with a 1:1 mixture of rhesus and human AB sera and rhesus erythrocytes (14). A combination of rhesus and human sera was also effective in cultivation of *P. gonderi* (24). Wickham et al. (97) used rhesus or kra erythrocytes with human serum for cultivation of *P. knowlesi*, and Nguyen-Dinh et al. (62) used rhesus blood cells for *P. cynomolgi*. Human erythrocytes did not support development of simian malarial parasites (14, 24, 62), indicating host specificity. Cultivation was accomplished in candle jars in plates or multiwell plates and sealed flasks (24), as well as in a continuous-flow system (62). Petri plate cultures in a candle jar would not support growth of *P. inui* in HEPES-buffered RPMI 1640 with rhesus serum and erythrocytes (61). A continuous-flow system was successful, however, with stages of *P. inui* formed after 35 days in culture infective for monkeys.

Rodent malarias. Among the rodent malarias, the erythrocytic stages of P. *berghei* and *P. chabaudi* have been cultivated in vitro. Static cultures of *P. berghei* were compared to suspension cultures by Mons et al. (60). They postulated that the metabolic rate of *P. berghei* parasites may be greater than that of *P. falciparum*, leading to more rapid depletion of nutrients from the growth medium and accumulation of metabolic wastes. Thus, in static cultures, conditions may rapidly develop that lead to inhibition of parasite growth and development. Stirring may facilitate rupture of erythrocytes as in passage of infected cells through host capillaries. Mons et al. (60) suggested that a limiting factor in development of *P. berghei* may be a lack of sufficient reticulocytes for the parasite to invade (see growth of *P. vivax* above). The use of BME basal medium or BME plus William's medium E gave better growth of and incorporation of radioactive amino acids into *P. chabaudi* than did RPMI 1640 medium (63). No growth of simian or rodent malarias has been accomplished in cell-free systems.

Avian malarias. Among the avian malarias, the erythrocytic stage of *P. lophurae* has been cultivated in vitro. Parasitic stages of *P. lophurae* freed from host cells by immune lysis were grown extracellularly in duck erythrocyte extract (46). Degenerate forms of the parasite, reduced incorporation of labeled amino acids, and decreased number, size, and density of food vacuoles appeared with time (about 3 days) in cultured forms.

Sporogonic Stages

Ookinete development. In vitro ookinete formation from in vivo-produced gametocytes of *P. berghei* in MEM and other tissue culture media was examined by Weiss and Vanderberg (96). Although MEM gave optimal results, the number of ookinetes formed was 1% or less of the number of macrogametocytes introduced. A better percentage of ookinete formation (up to 44%) was obtained by Janse et al. (34), in a study comparing HEPES-buffered RPMI 1640 and MEM media, from in vivo and in vitro-produced *P. berghei* gametocytes. RPMI 1640 gave consistently higher ookinete yields than MEM. Janse et al. also reported increased ookinete formation with decreased numbers of blood cells, suggesting that the microgametes had better motility with reduced cell density.

TABLE 3. In vitro cultivation of stages of non-human *Plasmodium* spp.[a]

Stage	Species	Basal medium(a)	Additional factor	Reference(s)
Exoerythrocytic	*P. berghei*	L-15	Rodent liver cells	19
		MEM; NCTC-135	Human embryonic lung or hepatoma cells	29, 30
	P. cynomolgi	MEM	Primary hepatocytes	57
	P. fallax	Medium 199-LDGM	Turkey embryos	16
	P. yoellii	MEM	Human hepatoma cells	12
Erythrocytic	*P. berghei*	RPMI 1640		60
	P. chabaudi	BME; William's medium		63
	P. cynomolgi		Rhesus erythrocytes	62
	P. lophurae	Duck erythrocyte extract		46
Sporogonic	*P. berghei* (oocysts)	RPMI 1640	With or without insect cells, fetal bovine, and silkworm sera	76
	P. berghei (ookinetes)	MEM		95
	P. gallinaceum	RPMI 1640	Fetal bovine serum plus *Drosophila* cells and Matrigel	93
	P. relictum (sporozoites and ookinetes)	Chicken serum and embryo extract		4, 5

[a] Summary of the different media used for cultivation of the several species of the malaria parasite and their different stages.

Syafruddin et al. (77) compared acellular and cellular environments for the production of *P. berghei* oocysts. Development from gametocyte to oocyst occurred in an acellular medium consisting of RPMI 1640 or Grace's medium with 10 or 20% fetal bovine serum or 5% silkworm serum (77). The presence of insect cells (*Aedes* or *Toxorhynchites* spp.) increased by ca. sixfold the number of oocysts produced compared to the acellular cultures. Cultures, however, did not develop beyond the oocyst stage.

Sporozoite development. Ball and Chao (5) studied in vitro development of sporozoites of *P. relictum*, an avian parasite, in a culture medium consisting of a phosphate- and bicarbonate-buffered basic salts solution, supplemented with glucose, amino acids, B vitamins, purines, and pyrimidines, plus chick serum and chicken embryo extract, in an atmosphere of 5% CO_2–95% air. High oxygen concentration was inhibitory for development of sporozoites (13). Development occurred from gametocyte to mature oocyst with sporozoites but ceased after five days. In another series of experiments (5), stomachs of mosquitoes which had fed upon infected canaries and contained immature stages of oocysts were dissected into a medium containing saline extract of pupae or adult mosquitoes at about 0.2%. Oocysts cultured for 48 or 96 h produced sporozoites infective for canaries, demonstrating that a period of residence in the salivary gland of the mosquito is not essential for development of infectivity of sporozoites.

Grace's insect tissue culture medium supplemented with various kinds of sera (fetal bovine, chick, and rabbit), as well as insect hemolymph, chicken embryo extract, and different types of adult and embryonic insect tissue extracts and cells, was used by Schneider (75) for in vitro development of sporozoites of *P. gallinaceum*, an avian parasite. Sporozoites were released from older (day 9), but not from younger oocysts. Warburg and Miller (93) used HEPES-buffered RPMI 1640 with 15% fetal bovine serum, supplemented with trehalose, hypoxanthine, and a lipoprotein-cholesterol mixture to obtain sporozoites of *P. gallinaceum*. Matrigel, a basement membrane-like extract containing laminin and collagen type IV, served as a substrate with *D. melanogaster* L2 cells and a gas phase of 5%CO_2–5% O_2–90% N_2. Under these conditions, transformation of ookinetes into oocysts with subsequent development of sporozoites took place. Without Matrigel, the parasites clumped and only a small percentage of ookinetes developed. Reviews of sporogonic development of *Plasmodium* are to be found in the works of Schneider and Vanderberg (76) and Vanderberg (93). Table 3 presents a summary of the media used in cultivation of life cycle stages of the non-human malarial parasites that are dealt with in the present review.

CULTIVATION AND DIAGNOSIS

In spite of the benefits to be had from cultivation of malaria parasites, it remains fundamentally a research technique. The difficulties associated with establishing *Plasmodium* spp. from humans or other vertebrates in vitro are a barrier to cultivation as a routine diagnostic tool. Medium preparation, sources of

sera and host cells (hepatocytes, erythrocytes, and reticulocytes), culture maintenance, and expense place cultivation within a research rather than a clinical diagnostic laboratory. Current techniques for diagnosis are well-established, with thin and thick smears being the most-reliable and -rapid and least-expensive technique for identifying parasites (71).

CONCLUSIONS

Trager and Jensen (88) retrospectively have examined how continuous cultivation of *P. falciparum* has affected malaria research. Drug screening and resistance, vaccine development, mechanisms of pathogenesis, development, genetics, and molecular and cellular biology are areas that have been impacted by use of in vitro cultivation of the malaria parasite. The availability of the technique has also spawned efforts to cultivate other malaria pathogens, both human and non-human. The ultimate goal is, of course, to cultivate the parasites in a totally defined medium, and as Trager and Jensen point out, the red blood cell is far from a defined medium. Thus, the next major goal, only partially realized, is growth of the malaria organisms free of their host cells.

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