PARASITE CULTIVATION IN RELATION TO RESEARCH ON THE CHEMOTHERAPY OF MALARIA

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INTRODUCTION

Publications dealing with the cultivation of malaria parasites commonly include the statement "these techniques have a direct application to studies on the chemotherapy of the disease". The obvious question to be asked is "have the authors' aspirations been fulfilled and does parasite cultivation have a part to play in the chemotherapy of malaria?" It is well known that we are some way from developing successful techniques for the continual cultivation of the malaria parasite; there are however, methods available for the short-term cultivation of all the stages of the life cycle. It is indicated to look at these methods in turn and to discuss the advantages and drawbacks in the application of these techniques to chemotherapy research.

Cultivation of the sporogonic stages of malaria parasites

Little attention has been paid to the cultivation in vitro of those stages in the life cycle which occur in the arthropod host. Although it is possible to maintain the different stages of the sporogonic cycle of Plasmodium relictum in the mosquito stomach, it has not been possible to obtain the complete cycle of a single culture isolation in vitro. The techniques involve tedious microsurgical procedures and maintenance of sterility is a major problem. It has now been demonstrated that ookinete formation in P. berghei can be obtained in cultures of both vector and non-vector cell lines, using primary cultures of Anopheles stephensi and epithelial cell line cultures of fathead minnow cells (Rosales-Ronquillo & Silverman, 1974). Ookinetes have been found growing intracellularly in fathead minnow cells in vitro (Rosales-Ronquillo et al., 1974). In these cultures the development of the parasite to the ookinete stage appears to be very similar to that described by Alger (1968), using a simple technique of incubating blood in capillary tubes at room temperature. None of these techniques have been developed quantitatively and it appears that studies on the cultivation of the sporogonic stages of malaria parasites have limited value in chemotherapy research. A simple technique such as that described by Alger could however be used to determine the viability of gametocytes after drug treatment.

Cultivation of the exoerythrocytic stages of malaria parasites

The exoerythrocytic stages of avian malaria parasites have been grown routinely in tissue culture for several years. Initially successful growth of P. gallinaceum was obtained by inoculating cultures with explants of infected tissue (Hawking, 1945). De Oliveira & Meyer (1955) and Meyer & Musacchio (1958) maintained a strain of P. gallinaceum in explants of...
embryonic brain and heart muscle for more than four years, growing the parasite alternately in plasma clots and hanging drops. These methods, although maintaining the parasite in vitro for considerable periods of time, required the addition of uninfected tissues to the cultures at periodic intervals and therefore had obvious disadvantages when compared with techniques subsequently developed from the work of Huff and his group. The success of these studies was due in part to the adaptation of the parasites to grow in embryos which provided a source of host cells and parasites for the cultivation in vitro (Huff, 1964). Parasites were grown in monolayers of primary embryo tissue cells and it was found at least with the P. falciparum turkey embryo system, that subcultures over several generations could be obtained. Large numbers of parasites were obtained in vitro and there was complete absence of erythrocytic stages from the cultures (Davis et al., 1966), an advantage when studying stage-specific effects. Exoerythrocytic stages must be maintained in turkey embryos in case subcultures fail. This system, with minor modifications, has been applied by Beaudoin for the study of the mode of action of antimalarial drugs. Beaudoin & Aikawa (1968), Beaudoin et al. (1969) and Aikawa & Beaudoin (1969) demonstrated that primaquine, naphthoquinone and RC 12 produce swollen mitochondria in the exoerythrocytic stages of P. falciparum grown in vitro, which suggests that these drugs may have their effect on oxidative phosphorylation and electron transport systems in the parasite. In addition they report that WR 69320, a dihydrofolate reductase inhibitor, produced nuclear changes in the exoerythrocytic stages of P. falciparum which were indistinguishable from those produced by pyrimethamine on the erythrocytic stages of the parasite. Studies on the effect of drugs on the exoerythrocytic stages of malaria parasites are presently limited to these systems which involve the use of avian malaria parasites as the model species. Furthermore, the mode of action or screening of drugs can be assessed only on the basis of morphological criteria. No attempt has been made to quantitate the effect of drugs in the way that radiotracer incorporation has been applied to the erythrocytic stages in non-nucleated erythrocytes. The reasons are obvious as selective depression of parasite metabolism cannot be measured since the exoerythrocytic stages are grown in nucleated, metabolically active cells which, like the parasite, are capable of incorporating radiotracers.

Quantitative methods of drug evaluation appear out of the question until more information is available on the biochemical aspects of the host-parasite relationship in the exoerythrocytic stages. Avian malaria parasites are known to differ from the mammalian species, both biochemically and physiologically, and they grow in different cell types. A method for the cultivation of the exoerythrocytic stages of mammalian malaria parasites is, therefore, essential.

One of the major problems in developing mammalian systems has been the difficulty in maintaining cultures of mammalian liver parenchyma cells in vitro. Recently research in the field of mammalian liver tissue culture has advanced a great deal. Long-term cultures started from isolated adult liver parenchymal cells have been shown to maintain some of the functions of the cells of origin, including the induction of tyrosine aminotransferase (Gerschenson et al., 1970), retention of specific cell antigens (Type et al., 1972), synthesis of albumin (Kaighn & Prince, 1971) and the retention of certain cytochemical characters (Williams et al., 1971). However, since these cells are dividing, unlike adult liver cells, they lack many of the functions of liver parenchyma cells in vivo (Lambiotte et al., 1972). Waymouth et al. (1971) have established permanent cell lines from livers of mouse foetuses of 16 days gestation in a completely defined medium, and one of these, FL83B, has been shown to exhibit striking morphological similarities to parenchyma liver cells, producing 12 mouse serum proteins including albumin and high density lipoprotein. Although this cell line exhibits characteristics of liver cells it is a transformed cell line and therefore not identical to adult liver cells in vivo. However it is not yet established whether the maintenance of complete hepatocyte function is a prerequisite for the successful growth of the exoerythrocytic stages of the malaria parasite in liver cell cultures in vitro.

Beaudoin et al. (1974) have been able to infect embryo mouse liver cell cultures with merozoites of the exoerythrocytic stages of P. lophurae and P. falciparum. These mammalian cell cultures were not characterized and were not completely homogeneous but development of the merozoites into mature schizonts was obtained in the parenchyma-like cells, and to a lesser extent in fibroblastic cells present in the cultures. These results with avian parasites in
cells of mammalian origin indicate that the growth of the exoerythrocytic stages of mammalian malaria parasites in vitro may be possible. As Beaudoin et al. (1974) point out, emphasis must now be placed on developing techniques that will provide sufficient sporozoites of a mammalian parasite with which to infect liver cells grown in vitro. At present there appear to be two possibilities. Cultures could be inoculated with sporozoites obtained either directly by sterile dissection of the mosquito, or from blood of the host inoculated half-an-hour previously with large numbers of sporozoites. Both methods have technical problems but the production of viable sporozoites may be aided by the observation of Vanderberg (1974) who demonstrated that albumin has a striking stimulatory effect on the motility of Plasmodium sporozoites in vitro. An alternative to using sporozoites as the inoculum is the production of infected liver cell cultures from heavily infected livers by the methods developed for normal adult cultures. Both approaches appear to be worthy of investigation.

One should consider which parasite model would be the best to develop for these types of studies. In several types of mammalian malaria, e.g. P. berghei and P. falciparum, the exoerythrocytic stages are self-limiting whereas in P. cynomolgi and P. vivax, the exoerythrocytic cycle is thought to occur alongside the erythrocytic cycle. Accordingly, even if it were possible to obtain development of the exoerythrocytic stages of P. berghei or P. falciparum, continuous propagation would require adaptation for continuous growth in vitro. The initial choice of any test system however, will not rest on what is the ideal system, but will depend on the availability of material for infecting the tissue cultures and also the cell specificity of the parasite. It is usually assumed, maybe correctly, that the exoerythrocytic stages must be grown in hepatic cell lines. If this is so, then the rodent model system appears the most likely possibility since liver cells of other species have yet to be cultured serially in vitro. However, as Beaudoin et al. (1974) have demonstrated the species specificity may not be absolute, it may be possible to grow the exoerythrocytic stages of mammalian malaria parasites in non-host liver cultures. It would be interesting to determine the extent to which the malaria parasite exhibits cell specificity for the growth of the exoerythrocytic stages as it may be possible to develop techniques for growing the parasite in tissue cell lines other than those derived from the liver. Even if a continually propagating cell line of exoerythrocytic stages of mammalian malaria parasites should be developed, further difficulties may still arise. It is well known that eukaryotic cell lines change their characteristics during continual growth in vitro and it is interesting to note that the exoerythrocytic stages of P. gallinaceum appeared to lose their infectivity to red cells after four years continual growth in vitro (Meyer & Musacchio, 1958). Therefore a continually propagated malaria parasite may prove not to be biochemically identical with that obtained in the host. However these are points only worth considering as and when these stages of the malaria parasite have been successfully grown in vitro.

Cultivation of the erythrocytic stages of malaria parasites

Although it is impossible to cultivate the erythrocytic stages of malaria parasites serially in vitro, the success achieved in growing parasites through one asexual cycle has led to a number of applications to chemotherapeutic studies. There are several methods available for studying the effect of drugs on the development of the parasite in vitro and the selection of a test system depends greatly on the type of investigation, economic circumstances and the environment in which the work is being performed.

Suspensions of parasitized erythrocytes have been used to study the mode of action of anti-malarial drugs in vitro since the early days of malaria research. The advantages of such tests are self evident. They minimize the variations in apparent drug response due to host differences including its immunological state, they require only small amounts of drugs and obviate the difficulties of following up test subjects in the field and they do not require experimental animals or human volunteers.

The first attempts to grow the erythrocytic stages in vitro were made by Bass & Johns (1912). They observed the development of P. falciparum to the schizont stage in a static layer of whole blood with glucose added. This very simple technique has been modified by Rieckmann et al. (1968) and Rieckmann (1971) and has become the most widely used test system.
for studying antimalarial action in vitro in the field. The test is based on the following
principle. Maturation of parasites in vitro is inhibited by the 4-aminquinolines and
dihydrofolate reductase inhibitors. The extent of the inhibition can be assessed by comparing
the number of schizonts in treated blood samples with the number found in control cultures.
There appears to be a good correlation of this test with in vivo tests, particularly concerning
sensitivity and RI and R11 types of resistance of P. falciparum to chloroquine (Peters &
Seaton, 1971; Colwell et al., 1972; WHO Technical Report Series No. 529, 1973;
Sucharit et al., 1974). Although this method has the considerable advantages of simplicity,
rapidity and economy, all of which are of extreme importance in the field where the supply of
chemicals, apparatus and trained personnel may be major problems, the method does have defects.
Only 72% of the parasites in undiluted blood and 51% in blood diluted with normal saline grew
to the schizont stage in control vials (Colwell et al., 1972). This is not an ideal success
rate and does not compare with the results that can be obtained by dilution of the blood in
commercial tissue culture media (Trigg, 1967; Phillips et al., 1972). The parasite inoculum
requires to be of low parasitaemia and of advanced trophozoite stage in order to obtain
sufficient schizont development for assessment. This is not surprising since the only
buffering capacity of the medium is provided by the serum. The delay in obtaining the mature
parasite in the blood can be a problem since collection of blood at the right time is not
always convenient, either for the patient or for the doctor. The method has now been
applied to studies of drug action on P. vivax, but with less success than with P. falciparum.
Powell & Berglund (1974) concluded that in terms of simplicity of quantitative assessment,
reliability and validity, the system was considerably less useful or satisfactory when
applied to P. vivax. The Rieckmann test utilizes 1 ml of blood per culture, and it would be a
great advantage in the field if a microtechnique, using drops of finger tip blood, could be
developed. Apparently attempts along these lines have been unsuccessful (WHO Technical Report
Series No. 529, 1973), but there seems to be no major reason why an adaptation of the microtitre
tray technique of Phillips & Wilson (personal communication) could not be successful.

One of the technical problems encountered with the simple culture systems used in the
field is that large amounts of lactic acid are produced by the parasite and result
in pH changes. The only way of reducing this problem is by diluting the suspension of
infected cells in a buffered growth media, a method which is routinely used in research
laboratories. This not only reduces the variability in parasite growth by controlling pH,
but adds many of the growth factors required by the parasite in vitro. Thus greater flexi-

bility is achieved in the type of investigation which can be performed. The only argument
for using unbuffered systems in the field is the difficulty in obtaining and using the media.
These are real problems but are not insurmountable. Commercial tissue culture media are
available which allow the growth of the erythrocytic stages of P. falciparum from the ring
to the schizont stage with more reliability than is obtained with the simple Bass & Johns
technique (Trigg, 1967; Phillips et al., 1972; Siddiqui et al., 1970). The major problem
of using many culture media in the field is the reliance on special gas mixtures, mostly
5% CO2/95% air for buffering the bicarbonate buffer system employed. It may be possible to
overcome this difficulty if the malaria parasite will grow in media with reduced NaHCO3 con-
tensts and the medium is additionally buffered with zwitterion buffers, e.g. TES,1 HEPES2
(Eagle, 1971; Siddiqui & Schnell, 1973). This could obviate the use of CO2 gas mixtures.

The cultivation of the erythrocytic stages in growth media has been used mainly as a
research tool although Phillips et al. (1972) and Phillips & Wilson (personal communication)
have grown P. falciparum in commercial tissue culture medium '199' for the screening of sera
from malaria patients in the field. Richards & Williams (1973) have combined the use of this
type of test in vitro with a test in vivo for drug assessment. Although this technique does
not appear to be widely used, it does have considerable advantages over straight tests in vivo;

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1 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
2 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
these advantages have been stated already (Bertagna et al., 1972) but are still worth reporting here; (i) the infected cell can be exposed to a controlled concentration of drug; (ii) the concentration of drug attainable in vitro may be much higher than is possible in plasma so that less active compounds may be assessed. Statistical correlations between physicochemical properties and biological activities then improve; (iii) if a drug is shown to be active in vivo but not in vitro an active metabolite is indicated; (iv) only microgram quantities of an active metabolite need to be isolated in order to demonstrate activity in vitro; (v) mechanisms of drug action can be investigated; (vi) metabolic pathways can be traced which may lead to the development of new drugs; (vii) considerable economies can be achieved when primates are used since several experiments can be conducted in vitro concomitantly with an experiment in vivo on one infected animal.

It is not possible to grow all species of malaria parasites in vitro with equal ease. Good growth from ring to schizont stage with subsequent reinvasion of uninfected red cells can be routinely obtained with P. knowlesi from the rhesus monkey (Anfinsen et al., 1966; Geiman et al., 1966; Trigg, 1969; Butcher & Cohen, 1971). P. falciparum can also be easily maintained in vitro from the ring to the schizont stage from both humans (Trigg, 1967; Phillips et al., 1972) and Actus monkeys (Siddiqui & Schnell, 1972). However, for good consistent invasion of uninfected cells to occur in vitro with P. falciparum, a subculture technique involving the addition of fresh red cells has to be adopted (Phillips et al., 1972). If the P. falciparum/Actus monkey system is employed fresh human red cells should be added to get relatively high rates of invasion and multiplication in vitro (Trigg, 1975). P. vinckeii can be grown in vitro from ring to schizont stage with some invasion of new red cells, but the multiplication of the parasite is not significant (Trigg, 1968; Coombs, personal communication). P. berghei is also relatively difficult to grow in vitro. Growth to the schizont stage can be obtained but no significant invasion or multiplication occurs at 37°C. However, small but consistent degrees of multiplication can be obtained by incubating the infected blood at 15°C (Smalley & Butcher, 1975). P. berghei however, has certain disadvantages as it tends to be asynchronous and therefore less flexible as a model for some studies in vitro. Growth of all species through the asexual cycle tends to be slightly slower in vitro than in vivo (Trigg, 1969).

The good growth of the malaria parasite through one asexual cycle in vitro permits drug action to be assessed by criteria other than morphological features. Techniques have been developed which measure the effect of a drug on the incorporation of radiotracers into parasite protein, RNA and DNA, and on carbohydrate metabolism (Polet & Barr, 1968; Gutteridge & Trigg, 1971; Trigg et al., 1971; McCormick & Canfield, 1972). These methods are now used both for screening and for studying the mode of action of drugs (Richards & Williams, 1973), and can include other parameters, e.g. measurements of osmotic fragility, Na+/K+ conc. and pH for monitoring the effect of the drug in vitro. For screening purposes it is usual to employ one tracer for the assessment of drug action, the choice of tracer depending on the mode of action of the drug to be tested. It must be remembered that biochemical assessment of drug action should be combined with morphological assessment as neither parameter is absolute in its own right. Richards & Williams (1975) reported that no morphological differences could be observed between P. falciparum parasites from untreated controls and from cultures of parasites treated in vivo with a single dose of either chloroquine or an amidinoare but that for all the treated samples the incorporation of radioactive leucine into parasite protein was reduced to levels not significantly different from that observed in normal, uninfected blood. Conversely, Gutteridge & Trigg (1971) noted an unchanged pattern for both the incorporation of 14C-algal protein hydrolysate into protein and 3H-adenosine into DNA in culture of P. knowlesi treated with pyrimethamine in which the development of the schizont stage parasites was abnormal. However, both biochemical and morphological criteria indicate abnormalities if 3H-orotic acid incorporation into DNA is used as the biochemical parameter for the action of dihydrofolate reductase inhibitors (Richards, personal communication). Again, all these techniques are dependent on maintaining the test species in the animal host as there is no way to continually propagate the parasite in vitro: even if we were possible to grow the parasite continually within the host red cell, the problem of continual replacement of host red cell arises. It is therefore, sensible to look at possibilities of adapting the erythrocytic stages to grow in cell lines which can be continuously propagated. If there is specificity of the erythrocytic stages of the malaria
parasite in the digestion of haemoglobin, this would favour attempts to grow the parasite in erythroid cell cultures which are capable of synthesizing haemoglobin (Friend et al., 1971; Swetly & Ostertag, 1974). But the note of Speer & Silverman (1974) reporting the growth of *P. berghei* to the schizont stage in Leydig testicular tumour cells may indicate that this specificity could be overcome.

Studies on the cultivation of the malaria parasite clearly provide techniques which may be used for studying the effect of antimalarial drugs on the parasite. They may also provide information which could lead to the development of new antimalarial drugs. The studies of Walsh & Sherman (1968), Polet & Barr (1968) and Gutteridge & Trigg (1970) on the incorporation of radioactive nucleosides into cultures of the erythrocytic stages of the malaria parasites showed that the parasite had a limited capacity to synthesize purine nucleotides de novo. This work led König and his colleagues to study the adenosinmonophosphate salvage pathway in *P. chabaudi* (Lukow et al., 1973); they were able to detect differences between the enzymes of the host and the parasite (Schmidt et al., 1974). An area for the possible development of antimalarial drugs is evident here.

One of the most interesting aspects of current malaria research is the mechanism of entry of the parasite into the red cell. Merozoite preparations can now be obtained in vitro (Dennis et al., 1975) and the studies of Bannister et al. (1975) and Dvorak et al. (1975) suggest that the mechanism of invasion of the red cell by the merozoite involves two receptors, one for attachment and the other for the invagination of the erythrocyte membrane to form the vacuole which will surround the parasite inside the host cell. Where more is known of the invasion process it is possible that it could become a target for antimalarial drugs. It is theoretically possible that drugs which stabilize membranes or modify the surface in some way, could prevent the invasion of the parasite. In addition, merozoites are surrounded by a surface coat which may be involved in the attachment of the parasite to the red cell. Any drug which either bound to or removed this coat could perhaps prevent invasion taking place.

In conclusion, therefore, it appears that although techniques have not been developed for the continual propagation of the malaria parasite *in vitro*, methods are available which can be extremely useful for screening and studying the mode of action of drugs. In addition there are several lines of investigation currently in progress which will hopefully facilitate new approaches to chemotherapeutic studies of malaria.

**SUMMARY**

Attempts at developing techniques for the continual *in vitro* cultivation of the malaria parasite have not yet been successful. It has not been possible to obtain the complete sporogonic development *in vitro*, although some progress was made using *Plasmodium relictum* and *P. berghei*. The cultivation of erythrocytic stages *in vitro* has successfully been established with *P. gallinaceum* in tissue explants. *P. fallax* could be grown in monolayers of primary embryo tissue cells (turkey). With the recent development of mammalian liver cell lines, prospects of the *in vitro* cultivation of erythrocytic mammalian Plasmodia are greatly improved. While it is still impossible to cultivate the erythrocytic stages of malaria parasites *serially in vitro*, they were successfully grown through one asexual cycle. This progress has led to a number of applications to chemotherapeutic studies, to the testing of new antimalarial drugs and especially to the testing of the susceptibility of *P. falciparum* to chloroquine. The method can be greatly improved by an appropriate choice of culture media; the addition of fresh red cells in a subculture system permits to obtain relatively high rates of invasion and multiplication. Apart from the obvious applications in the screening and evaluation of antimalarial compounds, the *in vitro* system lends itself to the study of the mechanism of entry of the parasite into the red blood cell.
On a cherché - sans succès - à mettre au point des techniques pour la culture in vitro continue du parasite du paludisme. L'auteur a obtenu certains résultats avec Plasmodium relictum et P. berghei, mais il n'a pas pu réaliser in vitro le développement sporogonique complet. La culture in vitro de parasites aux stades exoérythrocytaires a été bien réalisée avec P. gallinaceum sur des explants tissulaires. Il a été possible de cultiver P. falciparum sur des couches monocellulaires de tissu embryonnaire de dindon. La récente mise au point de cultures de lignées de cellules hépatiques de mammifères a beaucoup accru les chances de réussir à cultiver in vitro des Plasmodium exoérythrocytaires de mammifères. Bien qu'il soit encore impossible de réaliser en série la culture in vitro des stades érythrocytaires des parasites du paludisme, on est parvenu à les cultiver au cours d'un cycle asexué. Cette nouveauté a permis plusieurs applications à des études chimiothérapeutiques, la mise à l'épreuve d'agents antipaludiques nouveaux et, en particulier, la détermination de la sensibilité de P. falciparum à la chloroquine. La méthode pourra être améliorée considérablement par le choix approprié des milieux de culture; l'addition d'hématies fraîches à une sous-culture permet l'obtention de taux relativement élevés d'invasion et de multiplication. Outre les applications évidentes à la sélection et à l'évaluation de composés antipaludiques, le système de culture in vitro se prêtera aussi à l'étude du mécanisme de pénétration du parasite dans l'hématie.
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