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CULTIVATION OF HUMAN MALARIA PARASITES,(U)
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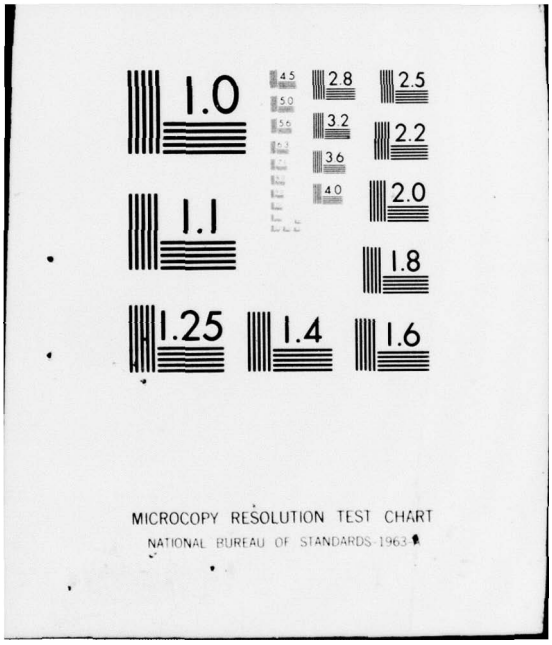
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(10) J. D. /Haynes, J. D. /Chulay, C. L. /Diggs, R. E. /Desjardins Cynthia /Hall

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(6) CULTIVATION OF HUMAN MALARIA PARASITES, (U)

(11) JUN 1978 (12) 8p.

J. D. /HAYNES, MAJ MC, *J. D. CHULAY, MAJ MC, C. L. DIGGS, COL MC, R. E. DESJARDINS, MAJ MC, and CYNTHIA HALL, SP5
WALTER REED ARMY INSTITUTE OF RESEARCH
WASHINGTON, D. C. 20012

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Malaria is a disease caused by protozoa of the genus Plasmodium, the blood stage of which causes fever, anemia, and other symptoms in infected humans. In every major armed conflict involving the United States, malaria has been a major cause of medical disability. Of the four species responsible for disease in man, Plasmodium falciparum is associated with the most illness. Because malaria is still widely distributed throughout Africa, Asia, Oceania, and Latin America, it remains the single most important potential medical threat to military operations. Since the discovery nearly 100 years ago of the causitive agent of malaria, efforts at control of this protozoan and its insect vectors have met with only limited success. Recent experience with increasing resistance of mosquitos to insecticides have made it clear that vector control is unlikely to eliminate this disease. Similarly, chemotherapy has failed in large areas of the world where P. falciparum has developed resistance to chloroquine and other drugs. Even in areas where drug resistance is not yet a problem, the difficulties in maintaining strict compliance with prophylactic regimens have resulted in failure to fully prevent disease.

Immunization would offer a logical means of protecting troops from acquiring malaria when entering endemic areas, but progress towards development of such immunization proceedures has been hindered by a number of problems. Natural immunity to P. falciparum develops slowly and is incomplete. No completely satisfactory animal model exists, and until recently malaria parasites could not be cultivated in vitro. These limitations made it impossible to prepare and test antigens which might potentially induce immunity. The

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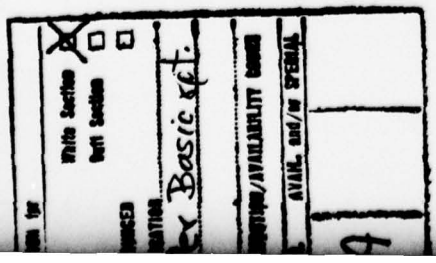
recent developments in our laboratory and at the Rockefeller University of methods for the continuous long-term culture of Plasmodium falciparum (1,2) make available a tool which can be used for studying pharmacologic, immunologic, and other biologic aspects of the host-parasite relationship. Antigens obtained from culture may eventually be used in preparing a vaccine against malaria.

The culture methods used involve mixing together infected and uninfected erythrocytes in buffered tissue culture medium, supplemented with human serum in a low oxygen, carbon dioxide-containing atmosphere, and incubating at 37 C. The infected erythrocytes can be obtained from humans, chimpanzees, or owl monkeys, and may be used immediately after collection, following cryopreservation, or from cultures. Uninfected erythrocytes can be from similar sources, although human erythrocytes stored using routine blood banking techniques are most convenient. Tissue culture medium can be either medium 199 (1) or RPMI 1640 (2), buffered with a bicarbonate-TES* or bicarbonate-HEPES* mixture. Human serum or plasma are equally effective at supplementing the medium, as long as it is obtained from a donor whose blood type is compatible with the erythrocytes used. The carbon dioxide content of the atmosphere can vary from 3 to 7%, adjusted to balance the bicarbonate in the medium to a pH of approximately 7.35 at 37 C. The oxygen content of the atmosphere can vary from 1 to 18%, but normal atmospheric oxygen (approximately 21%) is toxic for the parasite. The culture mixtures may be placed in tissue culture flasks which are individually flushed with the appropriate gas mixture, in microtiter plates which are placed in gas-tight boxes, or in petri dishes which are placed into a candle jar.

The current method which we use for maintenance of routine cultures consists of 5 ml samples with a 6% hematocrit in 25 sq cm tissue culture flasks containing M 199 buffered with 15 mM TES and 27 mM sodium bicarbonate, plus 10% heat inactivated human fresh frozen plasma. The flasks are flushed with a 5% carbon dioxide, 5% oxygen, 90% nitrogen mixture, sealed, and incubated at 37 C. Four ml of culture medium is changed daily, and samples for thin smears are made every 1 to 2 days. When parasitemia exceeds 1%, dilutions with fresh human erythrocytes are made to reduce the parasitemia to approximately 0.1%. Using this method, a 4 to 6-fold multiplication rate during each 48-hour cycle is obtained (figure 1). If the cultures are further diluted to 0.01%, fresh erythrocytes need not

*TES is N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, HEPES is N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.



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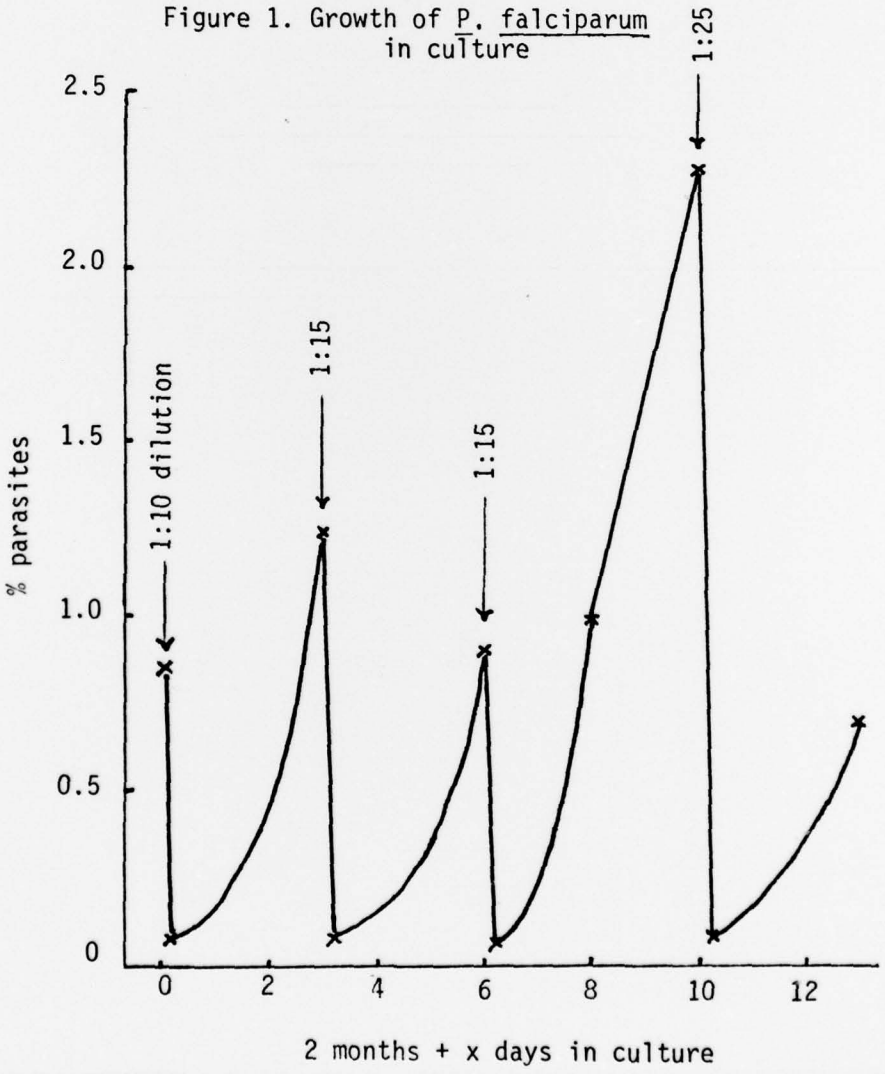
be added for 7 days or more. By changing the medium more frequently, or by reducing the hematocrit and adding an extract of erythrocytes, higher parasitemias can be obtained. When these two methods were combined, parasitemias as high as 33% were achieved. In addition to morphological evaluation of growth, the incorporation of a radioisotope (tritiated hypoxanthine) can be used as a rapid and sensitive method of comparing parasite growth under different conditions.

Figure 1. Growth of P. falciparum in culture



The in vitro cultivation system has found a wide range of application. When erythrocytes from different species of animals are used as uninfected target cells, only those from species which are susceptible to infection with P. falciparum in vivo are susceptible to in vitro invasion (table 1). Differences in erythrocyte receptors for different species of plasmodia were found by evaluating in vitro reinvasion of test erythrocytes by Plasmodium knowlesi , a simian parasite capable of infecting rhesus monkey and most humans, or P. falciparum, which is unable to infect rhesus monkeys. Humans with blood type Fy(a-b-), also known as Duffy negative, cannot be infected by P. knowlesi (3). The results of in vitro reinvasion corresponded directly with the known in vivo susceptibility, suggesting that the two parasites recognize different erythrocyte receptors (table 2).

Table 1. In vitro growth of P. falciparum in erythrocytes

test erythrocytes	% parasites day 1	% parasites day 3	multiplication rate/48 hr
human	.37	2.30	6.2
chimpanzee	.53	3.33	6.3
rhesus	.30	.13	.4
guinea pig	.55	.18	.3

(Adapted from reference 1)

Table 2. Differential invasion by P. falciparum and P. knowlesi

test erythrocytes	Parasites invading test erythrocytes (% of control human erythrocytes)	
	P. falciparum	P. knowlesi
human A+	100	100
human Fy(a-b-)	109	1
rhesus	1	277

(Adapted from reference 3)

Further evidence supporting this concept was obtained when normal human erythrocytes were treated with different enzymes prior to attempts at in vitro infection (table 3). Chymotrypsin treatment markedly inhibited invasion by P. knowlesi, but had no effect on the growth of P. falciparum. Conversely either neuraminidase or

trypsin treatment inhibited reinvasion by P. falciparum without affecting the growth of P. knowlesi. More knowledge about the parasite surface molecules responsible for parasite-erythrocyte interactions may aid in their purification. These molecules could then be used as antigens in a vaccine which might elicit anti-parasite antibodies acting to block the interaction of parasites with erythrocytes, thereby interrupting the parasite life cycle and preventing disease.

Table 3. Invasion of enzyme treated human erythrocytes

enzyme treatment	Parasites invading treated erythrocytes (% of untreated control erythrocytes)	
	P. falciparum	P. knowlesi
chymotrypsin	102	10
neuraminidase	46	109
trypsin	22	102

(Adapted from reference 3)

P. falciparum grown in vitro can be used to evaluate antimalarial drugs. If serial 2-fold dilutions of drug are made in the wells of a microtiter plate and aliquots of a culture are added to these, incubated for 24 to 48 hours, and then pulsed with tritiated hypoxanthine overnight, the effect of the drug on parasite growth can be assessed by morphology or by measuring incorporation of tritium into polynucleotides. One culture flask supplies enough parasites for 300 microtiter wells, which is sufficient to test in duplicate 7 different concentrations each of 18 different drugs. Semiautomated harvesting and counting devices yield raw data within a few hours. Using regression analysis, an estimate of the ED-50 (the dose at which there is 50% inhibition of parasite growth) can be obtained. When a strain of P. falciparum known to be sensitive to treatment with chloroquine in humans was tested and compared with a known chloroquine-resistant strain, a marked difference in the dose-response curves was apparent (figure 2). When mefloquine, a drug known to be effective for treating infections with either strain, was tested,

Figure 2.

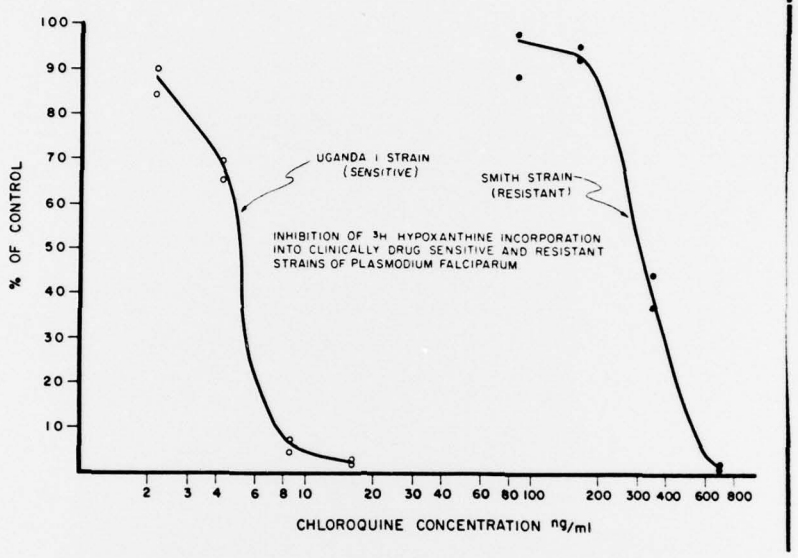


Table 4. Chloroquine sensitive and resistant P. falciparum strains evaluated in vitro

Drug	Concentration of drug (ng/ml) causing 50% reduction in uptake of tritiated-hypoxanthine.	
	Uganda strain	Vietnam strain
Chloroquine	8.8	184
Mefloquine	4.5	5.9

(Adapted from reference 4)

Current antimalaria drug screening tests generally are performed in vivo using animal models. These models are cumbersome because of the expense and manhours required, and in some cases yield data not applicable to human malaria. The method described here has the advantages of reducing screening time and expense, and uses only human malaria parasites. A spin-off of this research is that this method could be used to generate important information on the mechanisms of action of antimalaria drugs.

Cultured parasites may also be used to obtain antigen for a variety of immunological and serological tests. For example we have used these cultures as a source of antigen to detect antibodies using an indirect fluorescent technique. Thin smears made from culture were dried overnight, dilutions of test serum added, the slide thoroughly washed, flourescein-conjugated anti-human immunoglobulin reacted with the slide, and the slide washed and examined by fluorescence microscopy. Control sera from patients with no history of malaria gave titers no higher than 10, whereas the reference positive serum and sera from Africans living in an area endemic for malaria gave titers of 320 or higher (5). Similar titers were seen using either of two strains of P. falciparum taken from cultures 2 to 5 months old (table 5). This and other serologic techniques using antigen obtained from culture can be used in epidemiological studies of malaria transmission. Our results also demonstrate the ability to harvest antigens potentially important in the immune response to malaria.

Table 5. Indirect fluorescence antibody test using two strains of cultured P. falciparum as antigen

serum	IFA titer using Camp strain	Z strain
reference pos.	320	320
African 1	320	320
African 2	5,120	1,280
control 1	10	10
control 2	10	10

(Adapted from reference 5)

The culture system described here provides methodology for a variety of studies on the biology, immunology, and pharmacology of malaria. In addition to studies of parasite interactions with erythrocytes, evaluation of chemotherapeutic agents in vitro, and provision of antigen for serologic tests, once antigens are obtained in larger quantity from culture and purified, it should be possible to evaluate the feasibility of immunoprophylaxis against malaria.

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