Reports

Human Malaria Parasites in Continuous Culture

Abstract. Plasmodium falciparum *can now be maintained in continuous culture in human erythrocytes incubated at 38°C in RPMI 1640 medium with human serum under an atmosphere with 7 percent carbon dioxide and low oxygen (1 or 5 percent). The original parasite material, derived from an infected* Aotus trivirgatus *monkey, was diluted more than 100 million times by the addition of human erythrocytes at 3- or 4-day intervals. The parasites continued to reproduce in their normal asexual cycle of approximately 48 hours but were no longer highly synchronous. They have remained infective to* Aotus.

Prerequisite to the ultimate goal of a vaccine against malaria, a disease that still claims 96 million cases annually with a million deaths in Africa alone, is a method for in vitro propagation of the parasites (*1*). This has now been achieved.

Since the first attempts by Bass and Johns (*2*) and including all the more recent work with the erythrocytic stages of both human malaria and malarial parasites of experimental animals, only one or at best a few cycles of development in vitro have been obtained (*1, 3, 4*). Such short-term experiments have been useful in chemotherapeutic, biochemical, and immunological studies (*4, 5*). It was in experiments of this type (*6*) that the finding was made that medium RPMI 1640 (*7*), supplemented with Hepes buffer and monkey (*Macaca mulata*) serum, supported much better development of the monkey malaria *Plasmodium coatneyi* than did a variety of other media.

This medium with human serum was therefore tried in the continuous flow system previously found to support 4-day development of both *P. coatneyi* and *P. falciparum* (*4*). The principle of this method, which has been well described, is to have the erythrocytes in a shallow stationary layer covered by a shallow layer of medium that flows slowly and continuously over the settled cells. Two cultures were begun in the following way. Heparinized blood, from an *Aotus trivirgatus* monkey (*8*) infected with *P. falciparum* [FVO chloroquine-resistant strain obtained originally from Dr. W. A. Siddiqui (*9*)] and showing a parasitemia of 15 percent, was centrifuged, the plasma and buffy coat were removed, and the cells were resuspended in culture medium consisting of RPMI 1640 powdered medium (Grand Island) supplemented with Hepes buffer (25 m*M*) and 0.2 percent $NaHCO_3$ (hereafter abbreviated as RP) with 10 percent type AB human serum and with a volume of heparin solution (30 mg of heparin per 100 ml of blood in 0.85 percent NaCl) equivalent to one-tenth the volume of the resuspended cells. A suspension of normal human erythrocytes [type AB, Rh+ blood collected in acid, citrate, and dextrose (ACD) (*10*)] had meanwhile been prepared. Type AB blood was used because this or type B, but not type A, could be mixed with *Aotus* blood without danger of agglutination of the *Aotus* cells (*4*). The blood was centrifuged for 10 minutes at 1000*g*, the supernatant and buffy coat were removed, and the cells were suspended in an equal volume of RP and centrifuged again. This washing was repeated, and a 45 percent suspension of the washed cells was then made in RP with 15 percent type AB human serum (*10*). Eighteen milliliters of this suspension were mixed with 2 ml of the infected *Aotus* blood suspension. One milliliter of this mixture was placed in each of two flat-bottomed vials equipped with an overflow tube that is raised 3 mm above the bottom of the vial and delivers into an overflow collecting flask (*4*). Each vial was connected aseptically to a reservoir flask holding 120 ml of RP medium with 15 percent type AB serum. Both the vial and the reservoir flask received a slow current of a gas mixture consisting of 7 percent CO_2, 5 percent O_2, and 88 percent N_2. The flow of medium, controlled by a peristaltic pump, was started and adjusted to a rate of 50 ml/day.

At intervals of 2 days, or sometimes of 1 day, each vial was transferred to a new overflow flask and a new reservoir flask of medium was attached. At the same time, small samples of the settled erythrocytes were taken with a very fine capillary pipette and used for the preparation of wet mounts and a Giemsa-stained film. At intervals of 3 or 4 days, fresh hu-

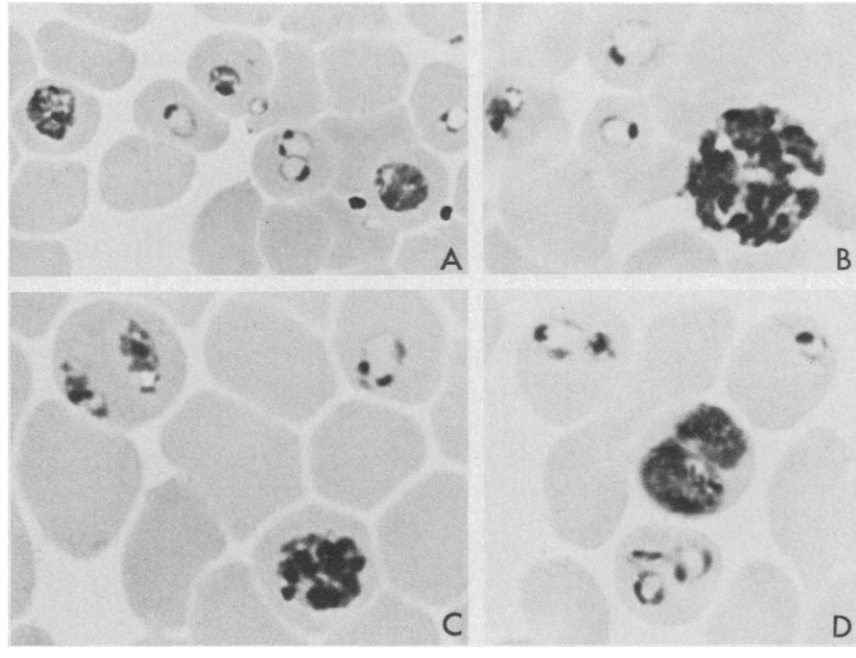

Fig. 1. *Plasmodium falciparum* after 50 days in continuous culture in human erythrocytes. (A) × 3500; (B–D) × 5400. Note all stages of the asexual cycle. In (B) one erythrocyte contains three late schizonts.

man AB erythrocytes, prepared in the following way, were added. An appropriate volume of blood in ACD (used after storage at 4°C for up to 2 weeks) was centrifuged, the supernatant and buffy coat were removed, and the cells were resuspended in an equal volume of RP and centrifuged again. This washing was repeated and the cells were finally resuspended at about 45 percent in RP containing 15 percent AB serum. Portions of this suspension—2, 2.5, or 3 ml, depending on the dilution desired—were then added to the culture vial and mixed thoroughly with the 1-ml content (see above) of the vial. The same volume that had been added was then drawn off, leaving in the vial 1 ml of blood now diluted 1 : 3 or 1 : 4 with fresh blood cell suspension. This procedure was tantamount to subculture, and the 2 to 3 ml of mixture that was removed could be used to start new cultures or for other experimental purposes. A droplet of the mixture was used to make a stained slide from which a parasite count was made. The number of parasites per 10,000 erythrocytes on this slide thus provided a base for estimating the extent of multiplication as seen in slides made on later days.

Representative counts were made (Table 1) for two lines (A, carried to 48 days, and B, still being carried as four sublines now more than 2 months in vitro). In line A the initial inoculum of young, ring forms grew into trophozoites and dividing forms in 2 days. At 4 days reinvasion had occurred but there was no increase in number. Nevertheless, the preparation was diluted threefold with erythrocyte suspension, reducing the parasite count to only six per 10,000 erythrocytes. Two days later it had risen to 38, most of which were rings. Such sixfold or better multiplication in a single cycle has continued (Table 1). In 50 days there would be 25 cycles. If we assume an average increase of only fourfold at each cycle, the overall increase in 50 days may be estimated as 4^{25}.

The gas mixture for line A was changed on day 36 to 7 percent CO_2, 1 percent O_2, and 92 percent N_2; this mixture was used throughout for line B. It appears to give better development in the flow vials than the mixture with 5 percent O_2. This low O_2 concentration was not strictly maintained because the cells were periodically exposed to air when being sampled and when being diluted with fresh erythrocytes.

Propagation of the parasites has also been obtained in simple plastic petri dishes held in a candle jar, with the medium being changed manually once a day. A candle jar is a long-known microbiological method (*11*) and has been used for the culture of parasites of the genus *Eimeria* in tissue culture (*12*). A white candle is centrally placed in a desiccator equipped with a stopcock. The candle is lit, and the cover is put on with the stopcock open. When the candle goes out the stopcock is closed. This is a simple but effective way to produce an atmosphere with low O_2 and high CO_2 content. On day 28 in vitro material (1.5 ml) from a flow vial was placed in 35-mm petri dishes (Falcon) to give a depth of about 2 mm. The dishes were placed in a candle jar and incubated at 38°C. Fresh medium was provided daily by gently tipping the dishes and withdrawing the supernatant fluid and replacing it with a portion of fresh RP containing 15 percent human serum. Fresh erythrocytes were added every third or fourth day as in the flow vial method. Excellent multiplication of parasites was observed. This method has the advantage of simplicity and the disadvantage of requiring daily attention for the change of medium. Both methods provide what may be the important requirements: a settled layer of erythrocytes in an appropriate culture medium, an atmosphere of relatively high CO_2 and low O_2, and a method for change of medium with minimal disturbance of the cells. The dish method has also been used to initiate a new culture line.

In the cultures all stages of the asexual cycle are readily seen (Fig. 1) at any one time, since the cultures have lost some of the synchronicity characteristic of *P. falciparum* in man (*13*) and also in *Aotus*. The parasites have retained altogether normal morphology. Delicate rings with two chromatin dots are common. Segmenters show 15 to 24 or more merozoites, the same numbers seen in material from human infections (*13*). Infectivity was demonstrated after 35 days in vitro by the inoculation of culture material intravenously into a splenectomized *Aotus* monkey. Parasites appeared in the monkey 6 days later, and the infection rose rapidly to a peak of 20 percent a week later when the monkey was killed. Only a few imperfect gameto-

Table 1. *Plasmodium falciparum* in continuous culture in human erythrocytes. Representative counts during the history of two lines. Line A was kept in flow vials continuously. Line B was derived from line A on day 28 in vitro and was passed through three successive petri dish cultures (with a four- and an eightfold dilution at subculture) before being returned to flow vials on day 38 after further fourfold dilution. Dilution refers to the dilution by volume of the culture with a 45 percent suspension of freshly washed human erythrocytes taken from cold storage in ACD (see text). At zero time a suspension of erythrocytes from an infected *Aotus trivirgatus* monkey was diluted tenfold with human erythrocyte suspension and this was considered the original undiluted concentration of parasites (32 per 10,000). Subcultures (that is, dilutions with fresh erythrocytes) were done at 3- or 4-day intervals and usually a fourfold dilution of the culture was effected. Abbreviations: R, rings without pigment; T, uninucleate forms with pigment; 2N, forms with two nuclei; and >2N, forms with more than two nuclei.

Days in vitro	Dilution	Parasites per 10,000 erythrocytes				
		R	T	2N	>2N	Total
		Line A				
0	1	32	0	0	0	32
2		6	23	7	17	53
4		18	5	0	7	30
4	3	1	1	0	4	6
6		27	7	2	2	38
11	36	5	2	1	1	9
13		19	13	3	3	38
14		40	9	0	1	50
22	1,728	9	2	0	1	12
24		30	20	3	6	59
25		29	32	6	15	82
28	18,000	11	3	0	0	14
31		34	25	3	4	66
46	10.8×10^6	3	1	0	0	4
48		22	5	2	2	31
		Line B				
28	18,000	11	3	0	0	14
31		54	14	2	7	77
35	580,000	6	3	0	0	9
38		56	9	3	1	69
38	2×10^6	5	1	0	0	6
41		29	9	3	1	42
46	32×10^6	8	1	2	0	11
50		170	48	28	46	292
50	128×10^6	7	5	3	2	17
53		144	76	8	14	242

cytes have so far been noted in preparations after 2 months in vitro, but sufficiently thorough examination of the slides for this stage has not yet been done. Whether infective gametocytes can be produced in vitro and what are the conditions for their formation are among the many problems now open to experimental attack.

Of more immediate importance is the use of the cultures for the preparation of merozoites, which may be particularly immunogenic (*14*), and for the study of materials produced by merozoites that may function in invasion of erythrocytes (*15*) and may have a role in induction of protective immunity. Of particular interest would be a study of the physiological condition of the erythrocyte, especially with regard to adenosine triphosphate content, in relation to its suitability for development of the parasites (*16*), and for characterization of the requirements of malaria parasites for extracellular development in vitro (*16*).

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17. Supported in part by NIH grant AI 10640 and mainly by AID contract ta-C-1200. We thank R. Klatt for technical assistance.

19 April 1976

Neurons Selective for Orientation and Binocular Disparity in the Visual Wulst of the Barn Owl (*Tyto alba*)

Abstract. *The visual response properties of single neurons in the owl's visual Wulst suggest that this forebrain structure is an analog of the mammalian visual cortex. Features in common with the cat and the monkey visual cortex include a precise topographic organization, a high degree of binocular interaction, and selectivity for orientation, direction of movement, and binocular disparity of straight-line contours.*

The frontal eyes and prey-catching skills of the owl suggest that the bird achieves stereopsis, the highly precise, binocular depth sense (*1*). However, some investigators (*2*) have argued against this possibility because owls lack partial decussation at the optic chiasm; instead, all the fibers from one eye cross to the opposite side of the brain (Fig. 1).

Partial decussation allows information from each eye to be compared by the brain, as Newton first pointed out (*3*), and is a prominent feature of the visual system of all the binocular mammals including man. Since owls lack a partial decussation, it was inferred that they also lack neurons that can be influenced by both eyes and, therefore, the disparity-sensitive binocular neurons thought to mediate the first stages of stereoscopic visual processing in the cat and monkey visual cortex (*4*).

Neuroanatomical studies on the owl (*5*) suggest that such inferences were premature; despite a totally crossed pathway from the eye to the thalamic relay nucleus, the owl has a bilateral projection from the thalamus to the Wulst, a prominent bulge on the surface of the forebrain (Figs. 1 and 2). The finding of a representation of each eye within this structure and other cytoarchitectonic similarities to the visual cortex of mammals, led Karten and co-workers (*5*) to hypothesize that the Wulst might play the role for the owl of the visual cortex in the monkey and the cat.

We have studied the visual response properties of 260 single neurons in the Wulst of the barn owl (*Tyto alba*). Our findings confirm the recent ideas of the neuroanatomists, for the physiological similarities of the Wulst to the visual cortex of the cat and monkey are more striking than the differences. In common with the mammalian cortex are a precise retinotopic organization, a high degree of binocular interaction, and neurons with requirements for stimulus orientation, direction of movement, and binocular disparity.

We used tungsten-in-glass microelectrodes and conventional extracellular recording techniques from a closed chamber. Anesthesia was induced with ketamine (12 mg per kilogram of body weight, injected intramuscularly) and maintained by intermittent injections through a Teflon catheter placed in the pectoral muscle. As the degree of eye

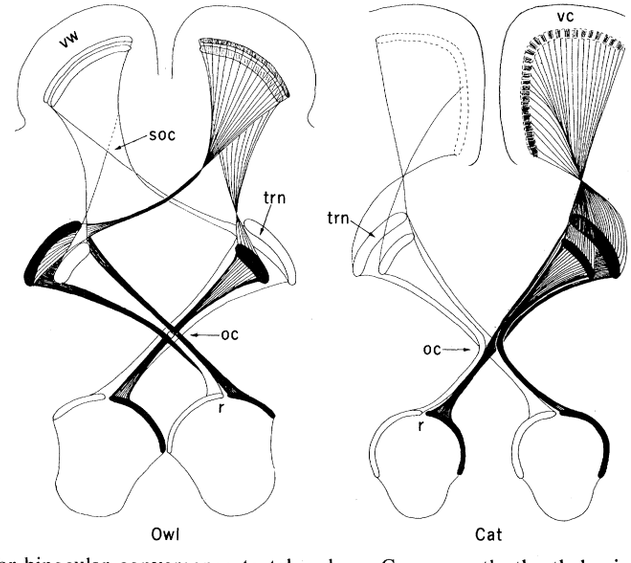

Fig. 1. Schematic representation of the organization of forebrain visual pathways in the cat and owl. Both of these vertebrates demonstrate binocular integration within laminated forebrain structures (visual cortex of the cat and visual Wulst of owl). In the cat (right) binocular convergence occurs at the thalamic level because of partial decussation of fibers from the retina (*r*) at the optic chiasm (*oc*). In contrast, the owl (left) has a total optic decussation, and thalamic fibers representing the temporal retina must recross in the supraopic chiasm (*soc*) for binocular convergence to take place. Consequently the thalamic relay nucleus (*trn*) of the owl represents the whole visual field of the contralateral eye while the corresponding relay nucleus of the cat represents the contralateral hemifield of both eyes. Despite these differences, both cat visual cortex (*vc*) and owl visual Wulst (*vw*) represent the contralateral hemifield, and both have binocular neurons with similar functional characteristics.