ditions used to examine pituitary binding. The specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ to a bovine pituitary nuclear site has characteristics similar to that previously described for vitamin D receptors in several target tissues (*3–6*). Most of the studies of vitamin D receptors in small intestine and bone have dealt with the interactions of the steroid hormone with cytosolic receptors and the subsequent translocation and binding of the hormone-receptor complex to a nuclear component (*5, 6*). In a report on binding of free 1α,25-$(OH)_2$-$[^3H]D_3$ to nuclear fractions from chick intestine and kidney (*7*), specific binding was a relatively small component of the overall binding. Our studies have revealed high-affinity labeling by free 1α,25-$(OH)_2$-$[^3H]D_3$ of receptors in the nuclear fraction of the bovine pituitary, with specific binding amounting to 85 percent of the total binding. Specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ to a cytosolic pituitary site in KCl-free medium was less extensive, but with 0.3*M* KCl present, binding to cytosol doubled and nuclear binding was abolished. This contrasts with the observation in the hen oviduct that under high ionic conditions (increased KCl), specific binding of progesterone hormone-receptor complexes to nuclear fractions is greatly increased (*15*). The kinetic constants for 1α,25-$(OH)_2D_3$ binding to the pituitary P_1 fraction are similar to those obtained for cytosolic receptors in rat bone cells (K_d = 0.2 n*M*; B_{max} = 75 fmole per milligram of protein) (*6*). However, in small intestine from vitamin D–deficient chicks, 1α,25-$(OH)_2D_3$ appears to label a much greater number of cytosolic receptors with a tenfold lower affinity (K_d = 2.2 n*M*; $B_{max} \sim$ 1700 fmole per milligram of protein) (*4*).

The present experiments demonstrating binding to the P_1 fraction do not differentiate between the direct interactions of 1α,25-$(OH)_2D_3$ with nuclear constituents and the exchange of 1α,25-$(OH)_2D_3$ with that already bound in hormone-cytosol receptor complexes. It is unlikely that the observed specific binding is due to interactions with plasma vitamin D binding protein retained in the tissue preparation, because at the plasma site 25-OH-D_3 is more potent than 1α,25-$(OH)_2D_3$, and the dissociation constant of 1α,25-$(OH)_2D_3$ is much greater than that found in the present experiments (*16*).

Although calcium-binding protein that is immunologically cross-reactive with the intestinal vitamin D–dependent calcium-binding protein is present in some brain areas, the brain protein does not appear to be rapidly induced by vitamin D treatment (*17*). The absence of specific nuclear vitamin D binding sites in central nervous system tissue in the present experiments is consistent with the unresponsiveness of brain tissue to vitamin D.

In preliminary experiments, we observed specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ to nuclear fractions from intact pituitaries of lactating rats and from cultures of rat pituitary adenoma GH_3 cells. Sites in these tissues show the same high-affinity kinetic characteristics as those found with the bovine pituitary receptors. Our observations are particularly interesting in view of the autoradiographic evidence (*18*) of 1α,25-$(OH)_2D_3$ binding to nuclei of cells of the rat pars distalis. Association of 1α,25-$(OH)_2$-$[^3H]D_3$ with a pituitary cytoplasmic site has been demonstrated in vivo in chickens (*19*). It has also been reported that 1α,25-$(OH)_2D_3$ may regulate protein phosphorylation and prolactin production in pituitary GH_4 cells (*20*).

HARRIS A. GELBARD
PAULA H. STERN
DAVID C. U'PRICHARD

Department of Pharmacology, Northwestern University School of Medicine, Chicago, Illinois 60611

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Cultivation in vitro of the Quartan Malaria Parasite *Plasmodium inui*

Abstract. *The simian quartan malaria parasite* Plasmodium inui *(OS strain) was cultured in a continuous flow system with rhesus monkey erythrocytes and RPMI 1640 medium supplemented with Hepes buffer and rhesus serum. Over a 10-week period, the growth of the parasite permitted a 61,000-fold cumulative dilution of the original inoculum. After 5 weeks in culture, the parasites were still infective to the monkey* Saimiri sciureus *and to* Anopheles freeborni *mosquitoes.*

The cultivation of malaria parasites offers a valuable research tool against a disease that still claims a heavy toll in many developing nations (*1*). Successful long-term cultivation has been achieved with the human malaria parasite *Plasmodium falciparum* (*2*) and the simian parasites *P. knowlesi* (*3*) and *P. fragile* (*4*). These species share common characteristics: they cause a severe disease in the infected host, and their erythrocytic asexual cycles have a short periodicity, with quotidian (24-hour) cycles for *P. knowlesi* and tertian (48-hour) cycles for *P. falciparum* and *P. fragile*. We now report the successful cultivation of *P. inui*, a parasite with fundamentally different characteristics.

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Quartan malaria parasites are distinguished by a 72-hour erythrocytic cycle and a usually more benign, chronic infection in their vertebrate host. This group includes the human parasite *P. malariae* and three simian counterparts, one of which, *P. inui*, is found in primates of the Old World (*5*). The OS strain of *P. inui* was isolated in India from a naturally infected *Macaca radiata* (*6*), and experimental studies have confirmed its quartan characteristics (*7*) and demonstrated its transmissibility to man (*8*).

Our initial attempts to cultivate this parasite by the petri dish–candle jar method (*9*) were unsuccessful. Parasites with normal morphology persisted for up to 34 days in culture, but failed to increase appreciably in number. However, these early experiments confirmed in vitro the quartan periodicity of *P. inui* (OS strain), since parasites that were started in culture at the ring stage matured synchronously into schizonts over a 3-day period.

We achieved the long-term cultivation of this parasite by using a modification of the continuous flow technique described by Trager (*10*). Heparinized blood containing 22,878 parasites per cubic millimeter was collected from an experimentally infected *Aotus trivirgatus* monkey (*11*). The parasitized blood was washed and the erythrocytes were resuspended to 50 percent in a culture medium consisting of RPMI 1640 medium (Gibco) supplemented with 30 m*M* Hepes buffer and 10 percent rhesus serum (*12*). Similarly prepared uninfected rhesus erythrocytes were used to dilute the parasitized erythrocytes threefold, and culture medium was added to adjust the cell suspension to 8 percent. Fifteen milliliters of the resulting culture material was introduced into a flat-bottomed vessel (*10*). A peristaltic pump (Harvard Apparatus) was used to maintain a continuous flow of fresh medium (60 ml/day), constantly renewing the culture medium overlying the settled erythrocytes. The culture vessel was gassed with a mixture of 7 percent CO_2, 5 percent O_2, and 88 percent N_2 (Matheson Gas Products). At 6- to 9-day intervals, one-half to three-fourths of the old culture material was removed from the vessel and replaced with an equal amount of an 8 percent suspension of freshly washed uninfected rhesus erythrocytes. This procedure, resulting in a dilution of the remaining parasites, was the equivalent of making a subculture.

Table 1. *Plasmodium inui* (OS strain) counts during the first 6 weeks in continuous culture.

Day of culture	Cumulative dilutions	Parasites per 10^4 erythrocytes					
					Schizonts		
		Gametocytes	Rings	Trophozoites	Two nuclei	More than two nuclei	Total
0		1	11	7	0	4	23
3		5	36	32	2	7	82
6		2	56	65	3	7	133
6	2	N.A.*	N.A.	N.A.	N.A.	N.A.	N.A.
10		2	50	6	1	1	60
13		3	65	46	6	13	133
13	4	0	30	32	0	0	62
20		14	145	45	4	11	219
20	16	2	44	7	0	4	57
28		11	67	59	1	2	140
28	64	3	27	10	2	1	43
30		4	55	16	3	5	83
34		4	61	40	4	0	109
34	128	0	30	18	0	0	48
40		3	62	14	1	7	87
42		2	62	35	4	11	114

*Not available.

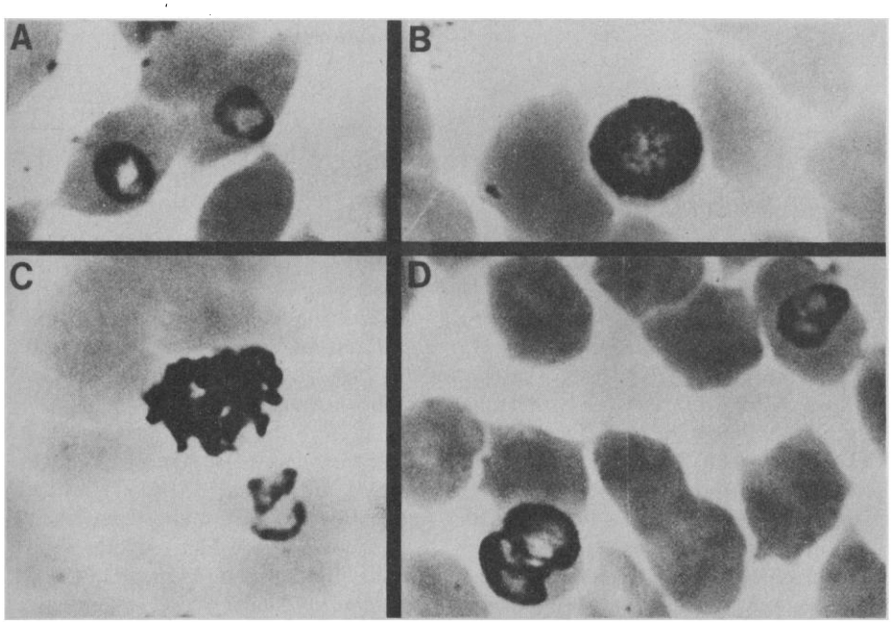

Fig. 1. *Plasmodium inui* (OS strain) in continuous culture. The parasites are shown at 28 days (140 parasites per 10^4 erythrocytes. (A) Rings, (B) male gametocyte, (C) schizont, and (D) trophozoites (× 2000).

The culture line was initiated at a parasite count of 23 per 10,000 erythrocytes; 6 days later there were 133 parasites per 10,000 erythrocytes, representing a sixfold increase over two asexual cycles. During the 6- to 9-day intervals between subcultures, the multiplication rate of the parasites varied from 2.5- to 6-fold, allowing the parasite count to reach values as high as 219 per 10,000 erythrocytes (Table 1). This growth rate compensated for the two- to fourfold dilution occurring at each subculture. After 10 weeks, when the culture line was discontinued, the original parasite inoculum had been cumulatively diluted 61,000-fold.

The growth pattern of *P. inui* in vitro differs strikingly from that of *P. falciparum*. In the latter, a tenfold multiplication in 2 days is not unusual, and parasite counts of 1,000 per 10,000 erythrocytes are easily obtained. While these differences are partially attributable to the longer cycle of *P. inui* and to the smaller number of merozoites produced in each schizont, other factors may be involved. Their identification would permit further improvement of the culture system.

The cultured parasites were morphologically normal and had, in Giemsa-

stained preparations, the same characteristics as *P. inui* found in infected monkeys. The ring stages had large chromatin dots, the trophozoite stages were typically amoeboid, and mature schizonts had 10 to 16 merozoites. Both male and female gametocytes were observed (Fig. 1).

Cultured *P. inui* retained its infectivity to monkeys. Parasites grown in vitro for 35 days were inoculated intravenously into a splenectomized *Saimiri sciureus* monkey (*13*). Eight days later, parasites appeared in smears of peripheral blood, and the animal developed the chronic, benign infection typical of this quartan parasite.

The infective potential of the gametocytes produced in vitro was assessed by feeding cultured parasites through a membrane to *Anopheles freeborni* mosquitoes. Five attempts resulted in one success. Mosquitoes fed on the 34th day of culture were found to have developed oocysts in their guts when dissected 11 days later.

This successful cultivation of a quartan malaria parasite offers promise for future successes with the human parasite *P. malariae*. The possibility of long-term culture of *P. inui* (OS strain), a parasite that can be studied experimentally in various species of monkeys and that is also transmissible to man, will enhance its value as a model of quartan malaria for immunological, biochemical, and ultrastructural investigations.

PHUC NGUYEN-DINH
CARLOS C. CAMPBELL
WILLIAM E. COLLINS
Vector Biology and Control Division, Bureau of Tropical Diseases, Center for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia 30333

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12. Trade names and commercial sources are for identification purposes only and do not constitute endorsement by the Public Health Service or by the Department of Health and Human Services.
13. C. C. Campbell (unpublished manuscript) recently demonstrated that *P. inui* (OS strain) could infect *Saimiri sciureus* monkeys.
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Electrophoretic Injection of a Fluorescent Dye into Giant Mitochondria and Mitoplasts

Abstract. *Studies of the electrical properties of giant mitochondria and mitoplasts with microelectrodes have indicated that there are no significant metabolically dependent membrane potentials. The internal location of the microelectrode has been confirmed by electrophoretically microinjecting the water-soluble dye Lucifer yellow CH into giant mitochondria or mitoplasts.*

Electrophoretic delivery of dyes has been used in electrophysiology to demonstrate the location of the microelectrode tip and to trace cellular processes or connections between cells. One of the first dyes used was Procion yellow M-4RS (*1*). Another group of fluorescent compounds of varied molecular weights has been used to study cell junctions after electrophoretic delivery (*2*). Recently the dye Lucifer yellow CH (dilithium 6-amino-2-[(hydrazinocarbonyl) amino]-2,3-dihydro-1,3-dioxo-1*H*-benz-[*de*]isoquinoline-5,8-disulfonate) was introduced for these purposes (*3*). This dye is highly fluorescent and crosses the neuronal membrane very slowly. Furthermore, Lucifer yellow CH has been shown to diffuse rapidly inside the cells and, at least in neurons, it does not seem to attach significantly to cellular structures.

In the present study Lucifer yellow CH was microinjected electrophoretically into giant mitochondria (*4*, *5*) and giant mitoplasts (*5*) isolated from the livers of mice treated with Cuprizone. The location of the tip of the impaling microelectrode is an important question since previous studies (*5*–*8*) have indicated that the potentials across the membranes of functioning giant mitochondria generally range from about 10 to 20 mV (positive inside) and are metabolically independent. These measurements are not in agreement with one of the major theories of oxidative-phosphorylation, the chemiosmotic theory (*9*), and the location of the electrode tip has been questioned (*10*).

Table 1. Summary of results of microinjection of mitochondria and mitoplasts. Numbers correspond to mitochondria injected.

Preparation	Fluorescence	
	+	–
Mitochondria		
Experimental	55	15
Control (injection at surface)	0	21
Removal of dye by passage of current	1	6
Mitoplasts		
Experimental	15	6
Control	0	7

In the previous studies (*5*–*8*) with microelectrodes of the electrical properties of giant mitochondria isolated from the livers of mice maintained on a diet containing Cuprizone (*4*), the impaled mitochondria have been found to be viable by assays which showed that their ability to accumulate calcium phosphate or phosphorylate adenosine diphosphate was unaltered (*6*, *7*). A number of experimental observations suggest that the impaling microelectrode tips are in the internal space of mitochondria: (i) The membrane potential, usually positive inside, reverses in sign in the presence of valinomycin and becomes dependent on the K^+ concentration of the medium (*5*). (ii) When the mitochondria are impaled with two microelectrodes, the electrical potentials induced can be measured with one microelectrode while current is passed through the other (that is, the two electrodes exhibit cross talk when inside the mitochondrion) (*8*). (iii) The measured resistances and potentials are approximately the same when the mitochondria are stripped of outer membrane and the cristae everted (that is, in mitoplasts) (*6*). (iv) A single potential is recorded when the microelectrode moves through the mitochondrion until it emerges on the other side (*6*).

In our experiments, we filled conventional microelectrodes with 27 m*M* or 65

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