found to have donor DNA from the same plasmid source, as described here and in our previous study (*1*).

Some in vivo instability of DNA of exogenous origin may be indicated by the fact that, whereas all positive fetuses in our earlier series had numerous intact copies of both marker genes, we now observe in one of the positive adults only partial sequences of a single-copy HβG gene. Other examples have been recently reported of retention of intact gene sequences in mice after injection into eggs of recombinant DNA including the rabbit β-globin (*12*) or HSV tk (*13*) genes. Retention of only rearranged or partially deleted sequences of other genes has also been shown (*14, 15*). In some instances, germ line transmission of the foreign DNA was observed (*12, 15*).

Of importance for further work based on gene injections into eggs is whether any of these newly introduced genes can function. We have demonstrated that it is possible to obtain mouse fetuses that synthesize a functional HSV thymidine kinase protein after pronuclear injection with PtkHβ1 (*1*). Others have shown that by using a fusion construct containing the structural gene for HSV tk and the promoter/regulatory region of the mouse metallothionein-I gene, viral enzyme expression could be detected in the eggs (*16*) and in adult mice (*13*). In another report involving injection of the rabbit β-globin gene (*17*), preliminary data suggestive of possible rabbit globin formation were presented, although evidence for actual presence of donor DNA was cited for only one of the mice.

With respect to the animal designated mouse Exp. 1, in which the HSV tk gene was present (Fig. 1B), we tested for viral thymidine kinase activity in liver homogenates with the ^{125}I-labeled deoxycytidine assay in the presence of tetrahydrouridine (*18*); 0.1 percent of the enzyme as viral-type would be detectable. No viral-specific enzyme activity was found (data not shown). Evidence for HβG-specific transcripts was sought in both experimental animals with HβG sequences by analyzing total liver messenger RNA's (mRNA's) with the S1-nuclease mapping technique (*19–21*) capable of detecting one copy of mRNA per cell. The diagnostic fragment of human β-globin–specific RNA (211 bases in size) was not found (data not shown) when a 3′-end-labeled Eco RI restriction fragment was used for hybridization; therefore, the occurrence of correct 3′ human β-globin-specific transcripts is unlikely. S1 assays on RNA's from hematopoietic organs, for example, spleen, were not performed in this series; therefore, the possibility of tissue-specific mRNA expression was not ruled out.

Our combined observations on mouse fetuses (*1*) and adults, following introduction of PtkHβ1 DNA into eggs, have thus disclosed instances in which foreign gene sequences are present (usually intact), fully functional (in one case), and transmissible (in another case) to progeny. These results support the expectation that introduction of foreign DNA early in mammalian development may prove useful for in vivo analyses of gene regulation in differentiation, genetic diseases, and malignancy.

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Plasmodium falciparum Gametocytes from Culture in vitro Develop to Sporozoites That Are Infectious to Primates

Abstract. *Gametocytes of two strains of the human malaria parasite* Plasmodium falciparum *have been produced in high density by means of a continuous-flow cultivation system. The gametocytes of these two strains infected a mean of 36 percent and 71 percent, respectively, of* Anopheles freeborni *mosquitoes that fed on a suspension of red blood cells containing the cultured gametocytes. Sporozoites harvested from the infected mosquito salivary glands were infective to the chimpanzee* (Pan troglodytes) *and the owl monkey* (Aotus trivirgatus).

The gametocyte stage of malaria represents the evolutionary link by which the microorganism developing in the vertebrate host undergoes sexual differentiation and infects the invertebrate host (mosquitoes). Recently developed techniques for cultivating *Plasmodium falciparum* in vitro allow studies on the sexual stage of this most malignant of the human malaria parasites. Our initial demonstration of the culture of gametocytes in vitro that could infect mosquitoes (*1*) established that biologically mature gametocytes would develop outside the primate. Subsequently, modification of the prime culture conditions (*2, 3*) and supplementation of the culture medium with adenosine 3′,5′-monophosphate (cyclic AMP) (*4*) or hypoxanthine (*5*) have been reported to enhance gametocyte production or maturation.

Although these studies have defined conditions conducive to the production of infective gametocytes, not all the morphologically mature gametocytes so produced have proved to be infective to mosquitoes. Similarly, not all mosquitoes with gut infections (oocyst stage) have consistently supported further maturation of the parasite to the salivary gland infection (sporozoite stage). We now report studies in which the complete extrinsic development of the parasite in anopheline mosquitoes has been repeatedly accomplished, producing sporozoites fully infective to two species of nonhuman primate hosts for falciparum malaria.

The Indochina III/CDC strain of *P. falciparum* (*6*) was cultured from a Lao refugee in August 1980. Gametocyte stimulation studies with this strain were performed during the initial 12 months of cultivation in vitro. The Tanzania I/CDC

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falciparum strain (*7*) was initially isolated from a U.S. tourist in October 1978, and portions of the patient's parasitized blood were preserved in liquid nitrogen before the parasite was adapted to culture in vitro and attempts were made to stimulate gametocyte production. Once adapted to cultivation in vitro by means of standard Trager and Jensen (*8*) techniques, the Indochina III/CDC and the Tanzania I/CDC strains were transferred to the continuous-flow cultivation system as modified from Trager (*9*).

After the culture was initiated, no fresh red cells were added during the 15- to 22-day duration of each experiment. Differential counts of asexual parasites and gametocytes were made daily on Giemsa-stained thin blood films. The density of the asexual parasites generally reached a peak between days 5 and 8, depending on the initial parasite density, with 6 to 20 percent of the red blood cells becoming infected; the density then declined to approximately 60 percent of the peak density. Stage 2 gametocytes (*10*) appeared between days 4 and 7, generally after the parasite density reached its peak. Typically, several successive waves of gametocytes (0.4 to 0.85 percent of red blood cells infected) appeared with progression to stage 4, yet without full morphologic maturation to stage 5 gametocytes (*11*). Maturation to stage 5 micro- and macrogametocytes (males and females, respectively) occurred only as the asexual density decreased to 2 or 3 percent, at which time the actual hematocrit in the flow vessel was less than 1 percent.

Anopheline mosquitoes, in lots of 100, were permitted to feed to engorgement (generally for 5 to 10 minutes) on water-warmed (39°C), bell-shaped membrane feeders (*12, 13*). Ten to 12 mosquitoes from each lot were dissected on days 7 to 10 after feeding to determine if there was a developing gut infection by the falciparum parasite. Experiments in which mosquitoes were fed on days 7 to 12 after initiation of the culture, before the appearance of fully mature stage 5 gametocytes, resulted only occasionally in mosquito gut infection (less than 10 percent of mosquitoes infected). Higher rates of mosquito infection were obtained on days 13 to 19 of the culture when fully mature gametocytes were present (Table 1). Attempts to predict mosquito infection by the presence of microgametocytes in the culture indicated that infectivity correlated closely with the presence, but not necessarily the density, of male gametocytes.

Comparative feedings of *Anopheles freeborni, A. stephensi,* and *A. gambiae* were performed during two experiments with the Tanzania I/CDC strain (Table 2). Although all three mosquito species were infected, *A. freeborni* consistently had a higher infection rate than the other two species, confirming the advantage of *A. freeborni* for infectivity studies in our culture system.

On day 13 or 14 after being fed three to five mosquitoes from positive lots were dissected to determine if sporozoites were in the salivary glands. For transmission studies, salivary glands from mosquitoes were dissected on a glass slide in 20 percent chicken serum in normal saline. The glands were dissected free of the mosquito, and then gently disrupted at 4°C by drawing the inoculum into a syringe through a 25-gauge needle. Motile sporozoites were counted in a hemocytometer, and the counts were later confirmed on Giemsa-stained smears (*14*).

Two splenectomized chimpanzees (*Pan troglodytes*) and three splenectomized South American owl monkeys (*Aotus trivirgatus*) were inoculated intravenously with an average of 20,000 sporozoites each. One chimpanzee was challenged successively with both Tanzania I/CDC and Indochina III/CDC (the second challenge, with Tanzania I/CDC, was conducted 4 months after curative therapy for the infection with Indochina III/CDC resulting from the first challenge). Each of the three sporozoite challenges of chimpanzees and one of three inoculations of owl monkeys resulted in patent falciparum infections (Table 3).

The infectivity of sporozoites of the Tanzania I/CDC and Indochina III/CDC falciparum strains to nonhuman primates confirms that morphologically intact and biologically competent gametocytes can be obtained from culture in vitro. In view of the high rates of mosquito infection that have been achieved in our continuous-flow system, it will now be possible to study the sporogonic stage of *P. falciparum* malaria without using primate surrogate hosts. The recent advances in the development of a sporozoite vaccine

Table 1. Infection of *Anopheles freeborni* by gametocytes of two strains of *Plasmodium falciparum* cultured in vitro.

P. falciparum strain	Number of experiments*	Mean percentage (range) of mosquitoes infected	Geometric mean number of oocysts per infected mosquito	Maximum number of oocysts per infected mosquito
Indochina III/CDC	10	36 (17 to 72)	2.1	6
Tanzania I/CDC	6	71 (52 to 100)	7.9	43

*See (*15*).

Table 2. Infectivity of gametocytes of Tanzania I/CDC *Plasmodium falciparum* gametocytes to three species of *Anopheles* mosquitoes.

Anopheles species*	Percentage infected	Geometric mean number of oocysts per infected mosquito	Percentage of infected salavary glands
A. freeborni	75	11.3	70
A. stephensi	47	4.0	11
A. gambiae	39	3.9	15

*See (*16*).

Table 3. Results of challenging nonhuman primates with sporozoites dissected from *Anopheles freeborni* mosquitoes infected by cultured *Plasmodium falciparum* gametocytes.

Primate species	*P. falciparum* strain	Prepatent period* (days)	Maximum asexual parasitemia (day)
Pan troglodytes	Tanzania I/CDC	9	6,479/mm^3 (4)†
	Indochina III/CDC‡	9	39,060/mm^3 (9)†
	Tanzania I/CDC§	10	992/mm^3 (4)†
Aotus trivirgatus	Tanzania I/CDC	Never patent ‖	
		Never patent	
		29	10,600/mm^3 (48)

*See (*17, 18*). †The animal was treated on the day of maximum asexual parasitemia. ‡See (*19*). §See (*20*). ‖ See (*21*).

will be greatly facilitated by a reliable supply of *P. falciparum* sporozoites. Falciparum sporozoites will also be required to accomplish the cultivation of the remaining link in the complete transmission cycle of the human malarias, the exoerythrocytic stage which occurs in vivo in the hepatocyte.

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14. Quantitative sporozoite counts on fixed smears were performed according to the method of Earle-Perez [W. C. Earle and M. Perez, *J. Lab. Clin. Med.* **17**, 1124 (1932)].
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16. *Anopheles freeborni* is the reference CDC anopheline for malaria transmission studies. Our colony has been maintained since establishment in 1944 [N. F. Hardman, *J. Natl. Mal. Soc.* **6**, 165 (1974)]. *Anopheles stephensi* was originally obtained from R. Gwadz, National Institutes of Health, Bethesda, Md. *Anopheles gambiae* (KWA and G3 strains) was from the colony maintained by G. Davidson, London School of Hygiene and Tropical Medicine.
17. The prepatent period for sporozoite-induced *P. falciparum* infections in the chimpanzee is as short as 6¾ days [R. S. Bray, *Am. J. Trop. Med. Hyg.* **7**, 20 (1958)]. For a West African falciparum strain, Collins *et al.* (*18*) found a mean prepatent period of 19 days in *Aotus*, although a greater number of sporozoites was inoculated than we used for these chimpanzee infections.
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19. Hypoxanthine (50 μg/ml) was added to the culture medium for the entire cultivation period of this experiment. Two parallel experiments comparing RPMI-1640 medium with hypoxanthine supplementation (50 mg/liter) as proposed by Ifediba and Vanderberg (*5*) and without supplementation failed to demonstrate differences in gametocyte production or mosquito infection resulting from hypoxanthine.
20. Indochina III/CDC and Tanzania I/CDC parasites cultured in vitro from the infected chimpanzees retained their sensitivity patterns to chloroquine and pyrimethamine in vitro.
21. Studies of *P. falciparum* sporozoite infection of *Aotus* by Collins *et al.* (*18*) demonstrated a patent infection rate of 43 percent and indicated that not all monkeys of this primate species could be expected to develop a patent infection.
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Myocardial Injury: Quantitation by Cell Sorting Initiated with Antimyosin Fluorescent Spheres

Abstract. *Spheres coated with antibodies specific for myosin were used to detect myocardial cell membrane disruption by scanning electron microscopy. Injury in a population of cultured myocytes was then followed and measured by fluorescence-activated cell sorting. This approach provides a unique method for quantitating the evolution of myocardial injury and potentially for assessing the efficacy of interventions aimed at myocardial protection.*

Irreversible ischemic injury of cardiac myocytes results in loss of cell membrane integrity and leakage of intracellular enzymes (*1*). There is also a concomitant inward diffusion of extracellular macromolecules (*2*). For this reason, radiolabeled antibodies to cardiac myosin (AM) will enter the regions of myocardial necrosis and bind specifically to intracellular cardiac myosin (*3*); areas taking up antibody correspond histologically and histochemically to regions of myocardial necrosis (*4*). The application of this technique to the quantitation of myocardial cell necrosis has now been employed in conjunction with fluorescence-activated cell sorting to develop a model system for separating viable cells from dead ones. Such a method permits measurement of the effects of interventions aimed at myocardial protection.

Murine cardiac myosin was purified from a batch of 100 adult CD-1 mouse hearts by the method of Katz and coworkers (*5*), as described previously for the purification of canine cardiac myosin (*2–4*). New Zealand White rabbits were immunized with the purified murine myosin in Freund's complete adjuvant (*2*) and serum was obtained at 6 weeks or later. Immune antiserums detected ^{125}I-labeled cardiac myosin at dilutions of 1:100,000. Antibody to murine cardiac myosin (AMM) was purified from immune serums on a murine cardiac myosin–Sepharose affinity resin (*2*). The affinity-purified AMM was coupled to fluorescent Covaspheres (Co) (Covalent Technology Corporation) by addition of 100 μl of Co to 1 mg of AMM in 0.1*N* $NaHCO_3$, *p*H 8.3 (*6*). Bound and free AMM were separated by centrifugation in a Microfuge for 4 minutes at room temperature. The AMM-coupled Co (Co-AMM) were then washed twice with Dulbecco's minimum essential medium (DMEM) and stored at 4°C. Control Co preparations were prepared by coupling purified antibodies to a nonrelated molecule, alprenolol (Co-AA).

Neonatal CD-1 mice (Charles River Laboratories) were exsanguinated, and the hearts were treated with 0.25 percent trypsin-EDTA with vigorous stirring until the cells were dissociated. Murine liver cells were also obtained by the same procedure for control experiments. The separated neonatal murine myocytes were allowed to stabilize for 3 to 4 days in DMEM–10 percent fetal calf serum (FCS) (*7*). The beating myocytes were then incubated at 37°C for 24 hours in either high-glucose or glucose-free DMEM-FCS. Immediately following addition of high-glucose or glucose-free DMEM-FCS, each culture flask received either Co-AMM or Co-AA and was incubated at 37°C for 24 hours. The media were removed and culture flasks were washed twice with fresh media to remove unbound spheres. The cells were then treated with 0.25 percent trypsin-EDTA to separate the myocytes from the flasks; 3-ml portions of DMEM-FCS were added to inactivate the trypsin. Additional unbound Co were separated by centrifugation at 110*g* for 6 minutes through a layer of undiluted FCS. Cells with bound Co were recovered in the resulting pellet, which was resuspended

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