Use of non-human primate hepatocytes for *in vitro* study of the pre-erythrocytic stages of malaria parasites

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*Methods were developed that allow invasion of sporozoites from simian malaria parasite species (*Plasmodium cynomolgi, P. knowlesi, P. coatneyi, P. inui, P. gonderi, P. fragile*) and development to schizont stages in rhesus and* Saimiri *monkey hepatocytes. The* P. cynomolgi*–rhesus monkey model was used to study inhibition of schizont development using monoclonal antibodies (MAbs) produced against the circumsporozoite (CS) protein of various strains and species of malaria parasites. Immunoelectron microscopy, using gold-labelled MAbs and cultured parasites, demonstrated that the CS protein persists in 7-day old liver stages of* P. cynomolgi*, but is not expressed at the surface of infected hepatocytes. A rhesus monkey was immunized with autologous hepatocytes (collected by biopsy) infected* in vitro *with liver stages of* P. cynomolgi. *This immunization elicited antibodies reacting with sporozoite, liver stage, and blood-stage parasites. In addition, human malaria parasites (*P. falciparum, P. vivax, P. malariae*) have been cultured in* Saimiri *or rhesus monkey hepatocytes. The* P. vivax–Saimiri *monkey model was used to study inhibition activity of sera from* Saimiri *monkeys experimentally immunized with recombinant* P. vivax *CS proteins. Post-immunization sera inhibited the parasite development, thus demonstrating the induction of antibodies effective against sporozoites. No relationship, however, was detected between* in vitro *inhibition and* in vivo *protection or antibody titres determined by ELISA or IFA.*

Introduction

In the life-cycle of malaria parasites, exoerythrocytic (EE) stages constitute an essential link between the sporozoites inoculated by a mosquito bite and the blood stages responsible for symptoms of the disease. Investigations of EE stages are hampered by their short duration, and by the fact that in mammalian malaria these stages occur in a deep organ, the liver.

The study of EE parasites has been markedly enhanced by the development of techniques permitting their culture *in vitro*. Culture systems have been developed for rodent parasites (*1*) and for three species of human malaria parasites (*2–5*). However, rodent malaria species are not closely related to human malaria parasites, and host cells for use in *in vitro* studies on human malaria parasites are either difficult to obtain (human hepatocytes), or do not reflect the natural host–parasite relationship (hepatoma cells).

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As an alternative, simian malaria parasites provide several advantages. Rhesus monkeys (Old World monkeys) are readily available experimental animals, thus ensuring regular access to liver specimens and permitting comparisons between *in vivo* and *in vitro* results. Among the parasites infecting rhesus monkeys are some well-established models for human malarias. *Plasmodium cynomolgi* is a model for *P. vivax*; *P. inui* for *P. malariae*; *P. coatneyi* and *P. fragile* for *P. falciparum*. Additionally, the quotidian parasite *P. knowlesi* is frequently used for immunological studies on malaria.

Among human malaria parasites, *P. vivax* and *P. falciparum* infect *Saimiri* and *Aotus* monkeys (New World monkeys), and vaccine trials have been conducted using these animals. *In vitro* cultured EE stages of human malaria species can be used to evaluate sporozoite vaccine efficacy or to isolate liver stage antigens.

In vitro development of simian malaria parasites in primary cultures of simian hepatocytes will be presented here, completed by some research application of these cultures. In addition, development of human malaria species in simian hepatocytes will be presented, together with their application to *in vitro* evaluation of a *P. vivax* sporozoite immunization trial in *Saimiri* monkeys.

Methods and results

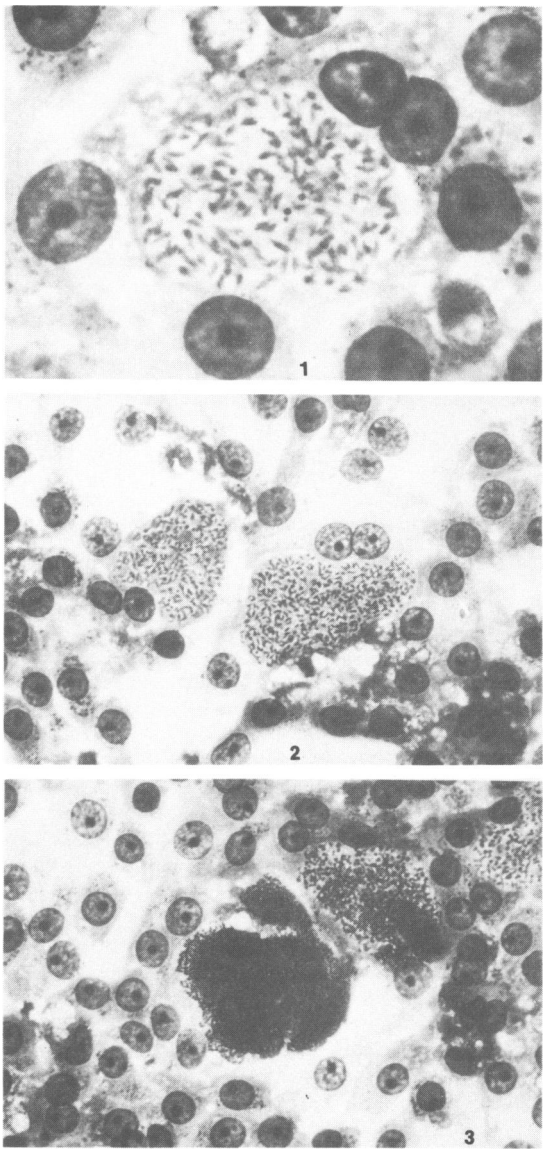
Primary cultures of hepatocytes were derived from liver biopsies of various simian species. A liver fragment (approximately 2 × 2 cm) was collected by laparotomy and perfused with a collagenase-Hepes buffer solution to dissociate the hepatocytes which were cultivated as previously described (*6*). One drop (25 µl) of the cell suspension, containing 30 000 to 40 000 hepatocytes, was deposited on each of 2 sites on the bottom of a 35-mm plastic tissue culture dish. Sedimentation, adherence, and resulting growth of the cells formed two disc-shaped (5-mm diameter) monolayers. Monolayers were infected with sporozoites obtained by dissection and grinding of salivary glands from infected mosquitos. As soon as the cells were spread out, the drop of medium covering the monolayer discs was removed and replaced with 25 µl of medium containing sporozoites. After 2 hours, the sporozoite suspension was removed and 1 ml of culture medium was added per culture dish, and changed daily. Cultures were incubated at 37 °C, in an atmosphere of 5% CO_2 and 95% air. At appropriate times, the monolayers were fixed with methanol then stained with Giemsa for observation of EE stage parasites.

Plasmodium cynomolgi (Fig. 1–3), *P. knowlesi*, *P. coatneyi*, and *P. inui* EE stages developed in primary culture of rhesus monkey hepatocytes (Table 1). From 1 to 80 schizonts were obtained per 10 000 sporozoites, depending on the experiments. In one experiment, a few *P. fragile* and *P. gonderi* EE schizonts were also obtained. *P. cynomolgi* and *P. coatneyi* EE schizonts were also cultured in *Saimiri* monkey (*Saimiri sciureus boliviensis*) hepatocytes. These findings are surprising since all attempts to infect these monkeys with sporozoites from these two species have been unsuccessful. Our *in vitro* findings suggest that the EE stages are able to develop in the liver, but that invasion into or development within erythrocytes may not be possible.

In terms of timing of development and morphology, the *in vitro* findings in all species correlated well with the *in vivo* results obtained in rhesus monkeys (*7–10*). Nuclear division and cytomerization were followed for *P. knowlesi* and *P. cynomolgi* schizonts. The *in vitro* timing of release of merozoites infective to rhesus monkey erythrocytes was in concordance with the *P. cynomolgi* and *P. knowlesi* prepatent periods occurring in rhesus monkeys. Use of an inverted phase-contrast microscope allowed observation of the evolution of *P. knowlesi* schizonts and of the burst of a mature parasite.

Based on previous studies (*11*), the *P. cynomolgi*–rhesus monkey model was used to study the inhibitory effect of anti-sporozoite monoclonal antibodies on the *in vitro* development of liver stages of *P. cynomolgi* (NIH strain of *P. cynomolgi bastianelli*) (*12*). MAbs against the CS proteins of five strains of *P. cynomolgi* (NIH, London, Gombak, Ceylon, Berok), and of *P. knowlesi* (H strain) were used. Incubation of *P. cynomolgi* (NIH strain) sporozoites with the anti-NIH strain MAbs totally prevented liver stage

Fig. 1–3. **Exoerythrocytic stages of *Plasmodium cynomolgi* in primary culture of rhesus monkey hepatocytes.** 1. 6-day-old parasite. 2. Immature 8-day-old parasite with still dividing nuclei. 3. Mature 8-day old parasite with rounded merozoites.



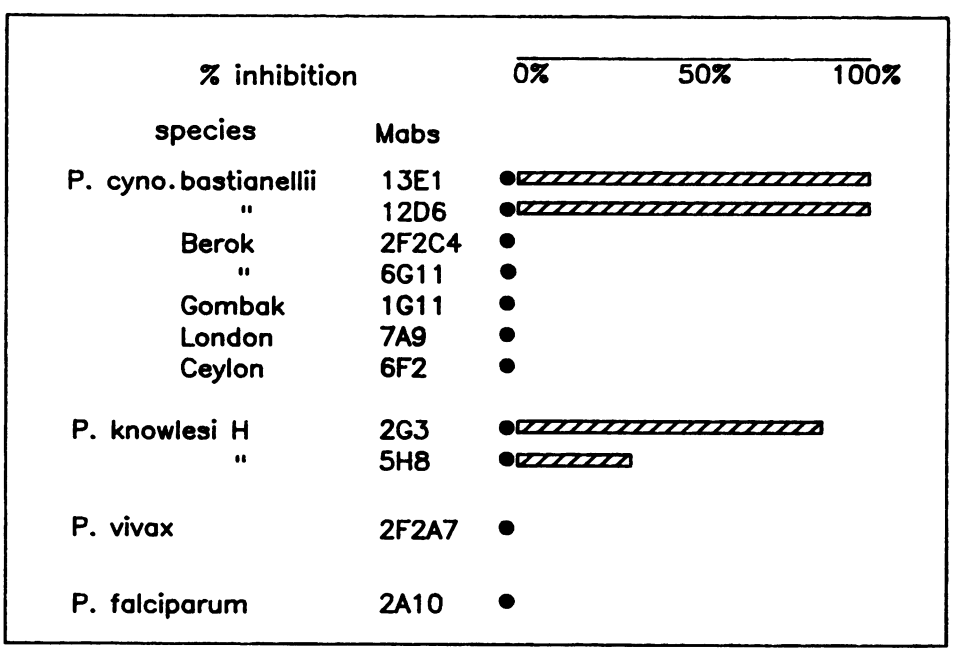
development; MAbs produced against the other four strains had no apparent activity. In addition, the anti-*P. knowlesi* MAbs had a partially inhibitory effect (80% inhibition) on parasite development (Fig. 4). These results confirm, in an *in vitro* functional assay, the CS protein strain specificity for simian malaria species, and the cross-reactivity between *P. knowlesi* and *P. cynomolgi bastianellii* previously demonstrated by other immunological methods (*13–15*). Complete inhibition of sporozoite invasion could be obtained only by using very high concentrations of antibodies. Antibodies against the CS protein had no effect when

Table 1: **Exoerythrocytic stages of simian and human malaria parasites established in primary cultures of simian hepatocytes**

Malaria species	Old world monkeys	New world monkeys		Apes
	M. mulatta	*S. sciureus*	*A.l. griseimembra*	*P. troglodytes*
Simian:				
P. cynomolgi	●[a]	●	NT	NT
P. knowlesi	●	NT	NT	NT
P. inui	●	NT	NT	NT
P. coatneyi	●	●	NT	NT
P. gonderi	●	NT	NT	NT
P. fragile	●	NT	NT	NT
Human:				
P. falciparum	●	○	○	NT
P. vivax	○	●	○	NT
P. malariae	NT	NT	●	●

[a] ● EE schizont development; ○ unsuccessful experiments; NT, not tried.

Fig. 4. **Percentage inhibition of *P. cynomolgi bastianellii* EE stage development by monoclonal antibodies directed against the CS protein of various strains of *P. cynomolgi*, *P. knowlesi*, *P. vivax*, and *P. falciparum*.**

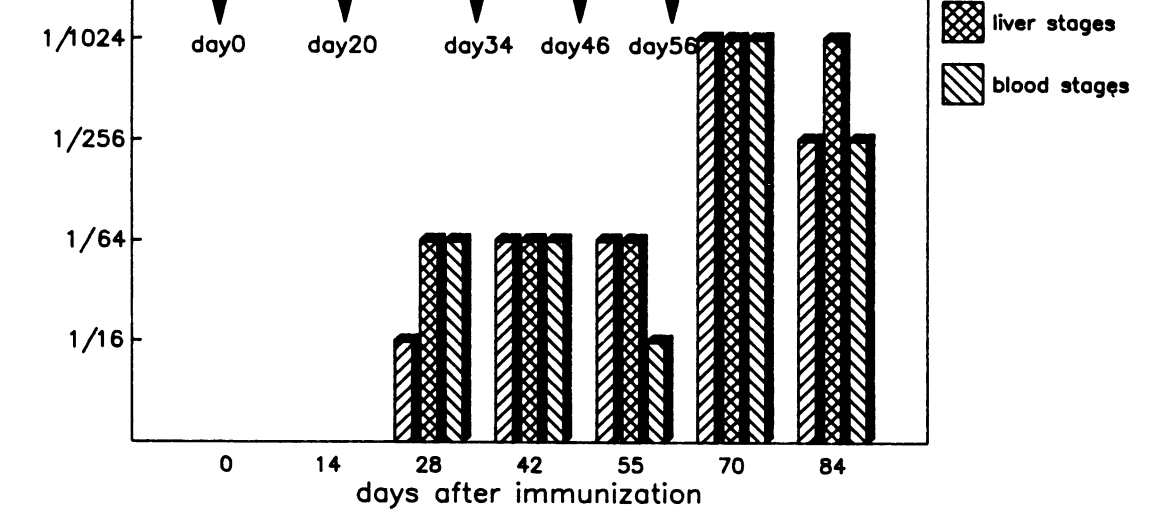


added during EE stage development. Thus it appears that antibodies against CS protein can play a protective role only by preventing invasion, and not by further inhibition of the EE stage development once a successful invasion has occurred.

Using the same *P. cynomolgi*–rhesus monkey model, immunoelectron microscopy localized CS antigen in 5- and 8-day old *in vitro* cultures of *P. cynomolgi* liver stages (*16*). A MAb (13E1) directed against the repeat region of *P. cynomolgi* CS protein densely labelled, in the presence of immunogold, the plasma membrane and surface of 5-day-old liver stage parasites as well as the surrounding parasitophorous vacuole membrane and space. Density of labelling decreased as liver stages increased in size and maturity. The CS antigen was not associated with internal structures within developing schizonts, or with the host cell cytoplasm or its surface. This indicates that components of the CS protein are not processed and expressed on the surface of infected hepatocytes, thus avoiding recognition by antibody or lymphocyte.

In order to determine the immunogenicity of EE stage antigens, a rhesus monkey was immunized with *in vitro* EE stages of *P. cynomolgi* cultured in autologous hepatocytes. Three intramuscular injections containing a total of 75 000 EE schizonts with muramyl dipeptide as adjuvant were given. A control animal was immunized with autologous cultured hepatocytes following the same procedure. Results of this study are shown in Fig. 5. Antibodies against the sporozoites, the EE stages, and the blood stages were obtained, but the monkey was not protected against sporozoite challenge. The control animal did not show any antibodies against sporozoites; antibodies against the blood stages were obtained only when parasitaemia was high. Immunoelectron microscopy using 7-day-old liver stages and the post-immunization serum previously adsorbed on uninfected hepatocytes showed antigenic distribution around the parasitophorous vacuole and inside the schizont, but not in the cytoplasm of the host cell. A liver biopsy was performed 7 days after sporozoite challenge, and liver sections were obtained. Many EE stage parasites were detected, none being surrounded by macrophages or other cells involved in the cellular response. However, a boost of antibodies reacting with the three stages was obtained after challenge. Western blot analysis performed with sporozoite and blood-stage extracts, and post-immunization sera showed that antibodies were produced against the CS protein, and five blood-stage proteins (data not shown). Such cross-reacting antigens have been identified for

Fig. 5. **Antibody titres against sporozoites, liver stages, and blood stages of *P. cynomolgi* following immunization of a rhesus monkey with liver stages of *P. cynomolgi* cultured in autologous hepatocytes and then challenged with sporozoites.** Immunofluorescence was done using air-dried parasites for all stages.



human malaria species by the use of *P. vivax* and *P. falciparum* anti-blood-stage MAbs (*17*, *18*). Our experiment shows that some of these proteins are in sufficient quantity in the liver stage to elicit an antibody response.

Another application of *in vitro* cultures in liver cells from non-human primates consisted in cultures of EE stages of human malaria parasites. *Plasmodium malariae* schizonts were obtained in chimpanzee hepatocytes (*19*). A few *P. malariae* schizonts were also obtained in *Aotus* monkey hepatocytes (*19*), while three attempts to grow *P. falciparum* failed. Surprisingly, hepatocytes from rhesus monkeys supported the growth of some *P. falciparum* schizonts (4 schizonts for 100 000 sporozoites).

Liver stages of *P. vivax* developed in primary cultures of hepatocytes from *Saimiri* monkeys (*Saimiri sciureus boliviensis*) (*20*), but not in *Aotus* monkey (*Aotus lemurinus griseimembra*) hepatocytes in spite of the fact that both of these New World monkey species are susceptible to *in vivo* infection by *P. vivax* sporozoites.

The *P. vivax–Saimiri* monkey model was used to correlate *in vitro* inhibition of liver stage parasites with immune response and immunoprotection in 18 *Saimiri* monkeys used in *P. vivax* vaccine trials using CS recombinant proteins (*21*). One recombinant ($NS1_{81}V20$) contained the repeat region of the CS protein. The other recombinant (VIVAX-1) contained the entire repeat domain and part of the surrounding regions. Both antigens were administered with aluminium hydroxide. In another group, six animals were immunized with irradiated sporozoites alone. Post-immunization sera from all immunized monkeys and from monkeys immunized with irradiated sporozoites inhibited parasite development when mixed with sporozoites, thus demonstrating the induction of antibodies effective against sporozoites, even by the non-protected animals (Table 2). However, 100% inhibition was rarely obtained, and no relationship was detected between *in vitro* inhibition and antibody titres detected by IFA or ELISA.

Conclusions

These studies have established that most of the simian and human malaria parasite species can develop in primary culture of simian hepatocytes. The *in vitro* cultured EE stages were used: (*a*) as functional assays to study the inhibitory activity of MAbs and sera against *P. cynomolgi* and *P. vivax* sporozoites; (*b*) for EE stage antigen production and characterization; and (*c*) for antigen localization (CS protein in the EE schizonts). Most of the studies conducted here focused on the CS protein of *P. cynomolgi* and of *P. vivax*. As in studies conducted by other authors (*22*), our results show a strong inhibition of liver stage development by antibodies directed against the CS protein of the parasites, but two points are not very encouraging for use of the CS protein alone as a vaccine: (i) a high level of antibodies did not often entirely inhibit the schizont development; (ii) immunoelectron microscopy studies did not show any expression of the CS protein at the surface of the infected hepatocytes. However, other malarial antigens may be expressed at the surface of the hepatocytes which may induce a humoral or cellular immune response against the parasite. *In vitro* models provide a tool for searching such proteins, and as direct functional assays for studying the humoral or cellular immune response to these antibodies.

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Table 2: **Summary of serological and parasitological results of vaccine trials directed against the CS protein of *P. vivax* using *Saimiri* monkeys, compared to the percentage inhibition of *P. vivax* EE stage development *in vitro* by sera from the immunized monkeys**

Antigen	Adjuvant	No. of animals	Serologic titres		No. protected	% inhibition of sporozoites *in vitro*[c]	
			IFA[a]	ELISA[b]		Range	Mean
Irradiated species	—	6	28 960	924	2 (33)[d]	84–100	96
VIVAX-1	Alum	6	5120	1055	1 (17)	87–100	95
$NS_{81}V20$	Alum	6	5120	2992	0	85–100	97
None	Alum	6	<20	<25	1 (7)	0–45	25

[a] Twofold dilutions of sera were tested in the IFA starting at 1:20 using glutaraldehyde-fixed sporozoites; IFA titres shown are for the day of challenge.
[b] Titres were expressed in OD units (the dilution of sera giving an optical density of 1.0).
[c] Percentage of inhibition compared to the pre-immunization sera activity for each monkey.
[d] Figures in parentheses are percentages.

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