Antigenic analysis of Plasmodium yoelii liver stages by fluorescence antibody assays

J. A. Londoño [1], *M. Sedegah* [2], *Y. Charoenvit* [2], *R. L. Beaudoin* [2], *P. Druilhe* [1]
[1]Medical Parasitology, Institut Pasteur, Paris, France; [2]Malaria Division, NMRI, Bethesda, MD, USA

Abstract

Little is known about the immune response against liver stage antigens which were first described for *Plasmodium falciparum*. In order to provide a basis for experimental studies, we analysed antigenically the liver stages of *Plasmodium yoelii* using sera of restricted specificity. Several distinct fluorescence patterns could be described in maturing liver forms. One pattern was identified as corresponding to antigens specific to the liver phase which are also species specific. Another pattern corresponds to sporozoite surface antigens which were predominant in liver trophozoites. Trophozoite-like liver forms were detected at least 7 days after the injection of irradiated sporozoites suggesting that parasites may persist and contribute to the immunity induced by this procedure.

Introduction

Antigenic analysis of *P. falciparum* liver forms by immunofluorescence has permitted to identify specific patterns of fluorescence corresponding to antigens expressed during the liver phase of development (Druilhe et al., 1984) and ultimately led to the characterization of individual molecules. In that study, a group of stage as well as species specific antigens was identified on morphological grounds in liver schizonts by using a series of sera containing antibodies with restricted stage specificities. The indications gained by polyclonal screening in the initial report were confirmed by the further identification and characterization of a gene coding for an antigen which, after careful analysis, could only be found in the *P. falciparum* liver schizont (guerin-Marchand et al., 1987). However access to primate malaria liver forms remains limited thus hampering extensive studies on this stage.

In order to allow the experimental studies required to evaluate the effect of the immune response to antigens specific of the liver phase, it was thought that the identification of similar antigens in a rodent malaria model would be useful. Previous studies have been performed using *P. berghei* liver forms (Danforth et al., 1978) but they only demonstrated the persistence of the circumsporozoite protein (CSP) in this stage and did not report on other antigens.

In the present study we used an approach similar to that formerly employed for *P. falciparum* to evaluate the specificities and immunogenicity of various groups of antigens found in *P. yoelii* liver forms.

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Materials and methods

Antigens

Sporozoites: Wet sporozoites preparations were made as described previously to permit the study of surface antigens (Druilhe et al., 1986). Briefly, salivary glands were dissected and homogenized in PBS and a drop containing about 500 sporozoites was deposited in each well of a poly-L-lysine coated antigen slide. Slides were then incubated at 4 °C to allow sporozoites to attach. Dried and acetone fixed sporozoite preparations were also used in parallel.

Liver Stages: Liver fragment biopsies were performed 10, 20, 35 and 44 hrs. following intravenous inoculation of 10^6 *P. yoelii* sporozoites (17X-NL strain) into either Dominion, A/J, CAF1, Balb/C or Swiss mice, or in gamma irradiated (800 rads) CAF1 mice. Biopsies were either fixed in Carnoy's then embedded in tissue tek and sectioned at 5 µm, or frozen in liquid N_2 and frozen 3 µm sections prepared and fixed in acetone prior to study. Biopsies were also taken from mice inoculated with 10^6 irradiated sporozoites (12,5 Krad, which did not produce a blood parasitemia) at 24 hrs and 7, 15 and 21 days and from mice immunized with 3 injections of irradiated sporozoites 7 days following a challenge with 10^6 sporozoites which was not followed by a blood parasitemia.

Erythrocytic Stages: Thin blood films were prepared from blood of mice with 20–30 % parasitemia with the 17X (NL) strain of *P. yoelii* and fixed with acetone.

Sera and antibodies

Mouse monoclonal antibodies (Mabs) directed against sporozoite surface antigens (NYS-1, -2, -3, -4, -5 and Mab 50:24:2, and 50:24:7) were prepared as described (Charoenvit et al., 1987).

Mouse antisera were pooled from 5 original samples collected 2 weeks after the last immunizing dose of animals immunized with one of a number of different parasite preparations, routes of inoculation, and vaccination protocols as follows:

Immunization with Sporozoite preparations

G1 Mice received intravenously 3 doses of 10^6 irradiated sporozoites (12.5 Krads) on a biweekly schedule.

G2 Same as G1, except that nonirradiated sporozoites were substituted for the irradiated ones and mice were put on chloroquine 24 h after sporozoite inoculation.

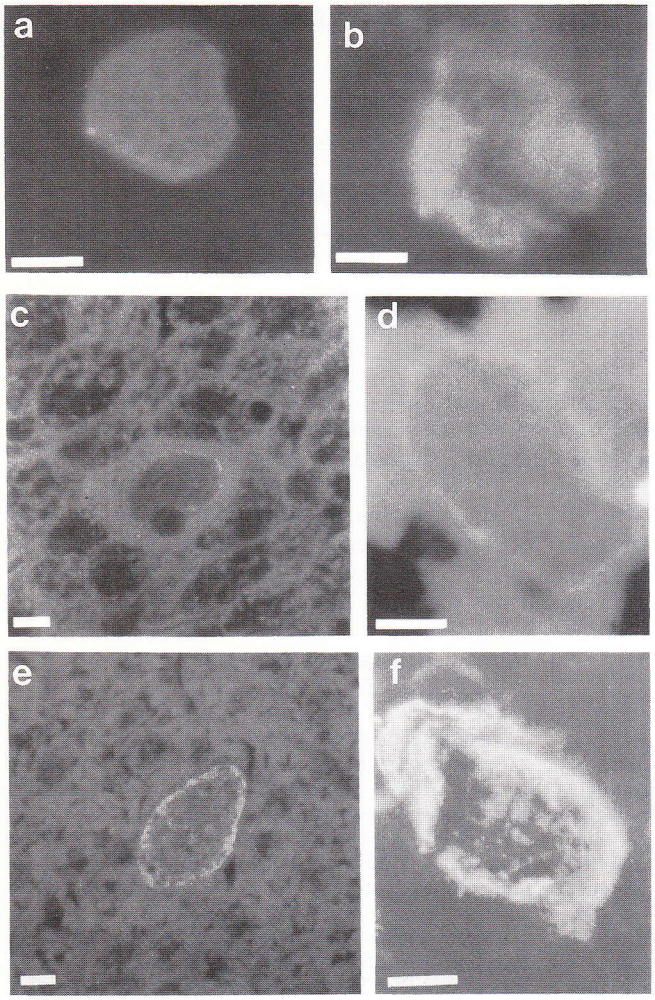

Fig. 1 Plasmodium yoelii liver schizonts as revealed by IFAT: Figs. A, C and E are 5 μm cuts from carnoy fixed and paraffin embedded liver fragments taken 44 hours after the IV injection of sporozoites. Figs. B, D and F are 3 μm cryo-cuts of N_2 liquid preserved liver fragments, fixed with aceton. Bars represent 10 μm. Figs. A and B show liver schizonts stained by G3 serum diluted 1/200 (mice received 3 × 10^6 normal sporozoites and developed blood infection). Fig. C and D show A pattern of fluorescence obtained with G1 serum diluted 1/200 (mice immunized three times with irradiated sporozoites) and 50:24:2 anti-sporozoite monoclonal antibody diluted 1/500, respectively. Fig. E and F show typical pattern B given by G2 serum 1/400 (mice received 3 × 10^6 normal sporozoites plus chloroquine, they did not develop blood infection)

G3 same as G2 only with no chloroquine, mice develop blood infection.
G4 Same as G1, except heat killed sporozoites (56°C for 10 min) were substituted.

Immunization with blood stages

G5 Pooled sera from mice bled 14 days after 5 iv inoculations at 2–3 wk intervals of 10^8 infected RBC's.

Immunization with liver stages

S62 Sera from mice receiving 3 IP inoculations, at 2 weeks interval, of liver cell homogenates containing 20 hr liver forms from an animal infected by 10^6 sporozoites.

Sporozoite induced infections

G052 2 iv injection of 5 × 10^3 sporozoites, at 15 day interval.
G52 2 iv injection of 5 × 10^4 sporozoites.
G502 2 iv injection of 5 × 10^5 sporozoites.

Heterologous Antibodies

Human sera were used which had been collected from

1) Three adult Africans living in an area holoendemic for *P. falciparum*.
2) Three European individuals 15 days after recovery from infections with *P. vivax*.
3) One individual following recovery from an infection with *P. malariae*.
4) One individual following recovery from an infection with *P. ovale*.
5) One individual following recovery from an infection with *P. cynomolgi* (human infection).

IFAT

End-point IFA titers were determined by testing 2-fold serial dilutions of the sera or Mabs on dried or wet sporozoite preparations, on liver schizonts in sections from Carnoy fixed or frozen liver biopsy fragments and on films of infected mouse blood. Depending on the serum tested, the second serum was an FITC labeled anti-mouse (Cappell Laboratories), or anti-human (Pasteur Production; IgG, A, M) diluted 1/100 in 0.02% Evans Blue solution. For liver sections an alternative method was to use for all dilutions and washings steps a blocking buffer consisting of PBS-Tween-milk (Tween 20 0.05%; non-fat powdered milk Regilait 1%). However, all titrations shown were carried out using the standard PBS 1% albumin buffer. Antigen slides were examined with a Leitz microscope equipped with epifluorescence, with a 50× oil immersion lens.

Results

No significant differences in receptivity to the liver stages of *Plasmodium yoelii* were observed between the mouse strains used, but more numerous liver forms per section were obtained when gamma-irradiated CAF mice were inoculated (data not shown).

IFAT using cryo-preserved and Carnoy fixed sections were found to be complementary as was the case in former *P. falciparum* studies (Druilhe et al., 1984, Galey & Druilhe unpublished) in that the reactivity of cryopreserved schizonts was greater, whereas parasite structure was better conserved in Carnoy fixed schizonts allowing more precise localization of the immuno-reactive structures (Fig. 1: A and B).

The various sera or antibodies used in IFAT allowed us to distinguish of 3 types of patterns on morphological grounds (Fig. 1: C to F):

A) A **thin peripheral ring**, smooth and regular, apparently corresponding to the outer membrane of the parasite (Fig. 1: C and D).
B) A **coarser peripheral ring**, with a smooth outer edge but rougher and more irregular on the inner edge, concentric with the thin ring described in (A). In mature forms this pattern became associated with labeling of the separations between cytomeres of the schizont, giving the appearance of a partitioning of the schizont (Fig. 1: E and F).
C) Various types of patchy, or cloudy, **diffuse labeling** of the internal part of the schizont but with no labeling of the periphery.

Table 1 IFAT titers recorded on *P. yoelii* sporozoites (SS), liver (LS) and asexual blood stages (ABS) with sera from *P. yoelii* immunized mice and human sera.
Titers were determined on wet preparations of sporozoites and on carnoy fixed-paraffin embedded liver cuts. IFAT liver stage patterns, A, B, C are described in text.

a) Homologous sera (P. yoelii)		SS	LS			ABS
			A	B	C	
Anti-SS						
G1		1600*	800	0**	0	0
G4		1600	200	0	0	400
Mab	50:24:2/50:24:7	40500	1000	0	0	0
	NYS1/NYS3	40500	1000	0	0	1000/4000
	NYS4	13500	1000	0	0	4000
	NYS2	4500	1000	0	0	1000
Anti-SS and LS						
G2		800	?***	3200	400	400
Anti-SS, LS, ABS						
G3		800	?	3200	400	1600
Anti-LS, ABS						
S62		50	?	100	200	1600
Anti-ABS						
G5		400	0	0	1600	48000
b) Heterologous sera						
P. falciparum	(1800)****	400	0	0	600	600
	(5400)	400	0	0	800	1800
	(600)	400	0	0	600	200
P. vivax	(1800)	200	0	0	600	600
	(5400)	400	0	0	1800	1800
	(1800)	400	0	0	600	600
P. malarie	(1800)	200	0	0	600	1800
P. ovale	(5400)	400	0	0	600	1800
P. cynomolgi	(1800)	400	0	0	600	600

* Reciprocal of the last positive dilution
** Negativity for the liver specific B pattern reflects negativity at a dilution of 1/50.
***? Presence of A pattern could not be established.
**** Titer on ABS of the corresponding species.

Differences in that general pattern were observed depending on the development of the schizont. The most striking one was a decrease of the reactivity of antigens corresponding to type A pattern from young to fully mature forms in which it became hardly visible, contrasting with a progressive increase in the amounts of antigens of type B pattern during schizont maturation, culminating with an increase in the number and reactivity of partitions throughout the mature schizont. In no case was specific labeling of the host cell cytoplasm or membranes observed. Some sera contained antibodies directed to nuclear components or induced some background fluorescence, most of which disappeared when the PBS-Tween-milk blocking buffer was used instead of the standard PBS-1% albumin.

Table 1a shows that the type A pattern of fluorescence corresponds to that produced with anti-sporozoite antibodies. G1 sera from mice protected against sporozoites, as well as 2 Mabs directed to *P. yoelii* sporozoites and non-reactive with asexual blood stages (ABS) show only this pattern on LS. The other Mabs directed to *P. yoelii* CS and to other sporozoite surface antigens, which crossreact to some extent with ABS (as well as G4 sera raised to dead sprozoites) also show pattern A on LS. In contrast, G5 sera from mice immunized with ABS, as well as all sera from heterologous infections, do not produce pattern A on *P. yoelii* LS. In the case of sera from G2 and G3 mice, the strong reaction obtained with antigens of pattern B (see below) did not allow us to determine whether or not they also react with antigens of pattern A.

Pattern B of reactivity occured only using sera from animals who harboured *P. yoelii* liver forms (G2, G3, G052 to G502). This pattern was more clearly distinguished at the last positive dilutions. In contrast, either homologous sporozoite (G1, G4 and Mabs) or blood stage induced antibodies (G5) never showed this type of pattern (G5 titrating to $1/5 \times 10^4$ against SIRBCs is negative on structure B of liver stages at 1/50) suggesting its stage specificity. Similarly, sera from individuals who have harboured liver forms of heterologous malaria species (and which reactivity by IFA with the homologous liver schizonts was established for *P. falciparum* and *P. vivax*) are non-reactive with that part of the *P. yoelii* liver schizonts (Table 1b), thus indicating the species specificity of the antigens responsible for pattern B. Conversely, G2 and G3

sera were negative with *P. falciparum* liver schizonts when reacted at 1/50 and above (data not shown).

The antibody titers to the stage-specific structure (B) were found to increase in response to increasing parasite loads (Table 2). They parallel titers of antibodies to sporozoites in animals injected 2 times with 5000 to 500,000 sporozoites. In contrast, in G2 and G3 animals injected 3 times with 10^6 sporozoites, higher titers are reached against the liver stage structure than against sporozoites or asexual blood stages (Table 1a).

All heterologous sera showed extensive cross-reactions with *P. yoelii* blood stages, liver stages as well as sporozoite stages even in the latter case when using an assay in which only surface antigens are accessible (Table 1b). Titers were generally lower on LS than on ABS, and moreover gave in all instances pattern C of fluorescence, as did G5 the anti-blood stage sera.

The heat-stability which was found to be a feature of *P. falciparum* LS specific antigens (Druilhe et al., 1984, Guerin-Marchand et al., 1987) was also investigated with *P. yoelii* LS: antigens corresponding to type C pattern were altered by heating at 100°C for 15 minutes while antigens corresponding to type B kept their antigenicity in the same conditions. Antigens corresponding to type A labelling appeared to be composed of two sets, one heat-stable revealed by 2 of the mabs studied and one which antigenicity was altered by heating, corresponding to the 3 remaining mabs.

The screening of biopsies from mice inoculated with 10^6 irradiated sporozoites permitted the detection of numerous intra-cellular small round forms about 3–4 µm in diameter. They showed a homogenous labelling in IFA assays using either anti-sporozoite Mabs (1/1000), or G1 serum (1/100) and either carnoy-fixed or cryo-preserved parasites. In giemsa-stained liver sections they appeared uninucleate, similar in shape and size to 10 hours liver forms of *P. yoelii*. They were detected in biopsies performed 48h, 4 days and 7 days after injection of irradiated sporozoites with some decrease in number, and were not detected in cotnrol mice receiving non-irradiated sporozoites and treated with chloroquine. Similar forms were also observed in mice receiving 3 inocula of irradiated sporozoites and challenged 21 days later with viable sporozoites, in blocks fixed 44h after the challenge.

No specific labeling of the cytoplasm or membrane of the host cells was observed using the antisera described here. In addition, no cellular inflammatory reaction could be found around the infected hepatocytes.

Table 2 IFAT titers of sera from mice injected with increasing number of normal sporozoites.

Sera	SS*	LS**	ABS
G 052	200	200 (200)	800
G 52	400	400 (200)	800
G 502	800	800 (400)	1600

*Titers were determined on wet sporozoites.
**Titers were determined on cryo-preserved sections. In brackets are the titers for the liver specific B pattern as determined on carnoy fixed fragments.

Discussion

On the basis of the reactivity of a series of sera raised to various development stages of the parasite we have identified in liver schizonts 3 distinct IFA patterns defining 3 distinct groups of antigens. One appears to correspond to sporozoite surface-associated antigens, one to liver stage specific antigens, and one to a wider group common to several stages of the homologous species, and to heterologous malaria species.

Liver-stage antigens belonging to pattern of reactivity A can be considered as associated with sporozoite surface antigens since they react, albeit at a lower titer, with antibodies having that specificity (i.e. G4 and Mabs).

Interestingly one Mab (NYS4) which gives a particular patchy fluorescence pattern on the sporozoite, likewise stains the liver stage with the identical patchy fluoresence pattern in contrast with other Mabs tested which IFAT pattern was regular. Moreover, the amount of such antigens decreases markedly as the liver schizont matures, suggesting that pattern A antigens are not synthesized during the liver phase of development and may correspond to residual sporozoite surface antigens following entry of the sporozoite into the hepatocyte. A similar type of antigen has been observed in *P. falciparum* liver forms except that it could be detected only in cryopreserved but not in Carnoy-fixed LS (Druilhe et al., 1984, and unpublished data). Similarly, in the *P. cynomolgi/Rhesus* monkey model, the CS antigen could not be detected in Carnoy fixed EE schizonts (Krotoski et al., 1981). Our observations on *P. yoelii* that the antigen reactive with anti-CS antibodies decreases throughout schizont maturation are in agreement with previous results obtained using *P. berghei* in rats (Danforth et al., 1978; Aikawa et al., 1981). However, results are not in agreement with in-vitro studies in which the CS reactivity remained unchanged both with *P. berghei* in the WI38 cell line (Hollindale et al., 1983), as with *P. yoelii* in rat hepatocytes (Mellouk et al., unpublished). A similar difference between in vivo and in vitro models was also noted using *P. falciparum* (unpublished results) and raises the need of performing comparative studies on the antigenic content of liver forms to determine the relevance of these models.

P. yoelii antigens corresponding to pattern B are of particular interest since by their stage-specificity, their species-specificity, their heat-stability and their location in the mature parasite, they closely resemble similar antigens observed in *P. falciparum* (Druilhe et al., 1984) as well as in *P. vivax* liver forms (Druilhe et al., unpublished data). For this reason their demonstration in the liver forms of a rodent parasite opens the possibility to study this new group of malaria antigens in laboratory hosts in order to determine their function and the biological effects of the immune response they may induce. In mice, the LS antigens of *P. yoelii* were found to be indeed immunogenic. High parasitic loads induced higher levels of antibody than did antigens of other stages. However, this difference was not as marked as was the case for the corresponding LS antigens from *P. falciparum*. High titers of antibodies to *P. falciparum* LSA could be detected in subjects receiv-

ing fewer than 5 infective mosquito bites, while a similar level of response required more than 10^6 *P. yoelii* sporozoites in mice. This may be related to the host-factors since laboratory mice differ from the African tree rats in which *P. yoelii* was identified and so do the course of infection. It may also simply be the result of the lower transformation rate of *P. yoelii* sporozoites to LS in mice (about 0.1%; data not shown) than of *P. falciparum* in primates (20 to 60%; Druilhe et al., 1984; Bray, 1958), or it could be possibly a reflection of the shorter time required for completing the liver cycle in the rodent parasite (44 hr compared to 7 days).

Finally, in contrast with EE schizonts produced in vitro (Hollingdale et al., 1983), we found that antibodies directed to blood stage antigens gave only a central diffuse staining of schizonts obtained from infected animals (Pattern C) and did not stain the parasite membrane.

The presence of apparently uninucleated liver forms in livers from animals receiving iradiated sprozoites confirm that at the dose used, irradiation do not kill the parasite which remains able to penetrate the hepatocyte and develop in liver trophozoite, as it was formerly found for *P. berghei* (Sigler et al., 1984). In addition these uninucleate forms remained unchanged and could still be detected at least 1 week after sporozoite inoculation. In view of the poor protection achieved by killed parasites injected IV or by irradiated parasites injected intramuscularly or subcutaneously (Cochrane et al., 1980), this observation supports the hypothesis that the strong immunity induced by IV injection of irradiated sporozoites may be due either to the antigen-depot effect of sporozoites antigens achieved by persistent uninucleate forms in the liver, or/and to the synthesis of novel, LS-specific, antigens, differing from sporozoite specific ones.

In conclusion, the rodent malaria do provide an experimental system which, when carefully probed, should yield answers to several questions about this ill-known stage of malaria parasites.

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Dr. Pierre Druilhe

Medical Parasitology
28, rue du Docteur Roux, 75724 Paris Cedex 15, France