Am. J. Trop. Med. Hyg., 33(3), 1984, pp. 336–341

SPECIES- AND STAGE-SPECIFIC ANTIGENS IN EXOERYTHROCYTIC STAGES OF *PLASMODIUM FALCIPARUM*

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Abstract. Numerous exoerythrocytic forms of *Plasmodium falciparum* (PFEEF) were obtained from the liver of the South American monkey, *Cebus apella*, for analysis of the antigens on this stage. As antigen for the fluorescent assay, 5-μm sections of liver fragments collected on day 5 following sporozoite inoculation and fixed in Carnoy's solution or kept in liquid nitrogen were used. Two types of fluorescent labeling of the PFEEF were identified: diffuse and peripheral. Each of 23 sera from individuals with *P. falciparum* infection acquired naturally by mosquito bite showed the diffuse and peripheral patterns of fluorescence at low serum dilutions (i.e., 1:10–1:100), but only peripheral staining at higher serum dilutions (i.e., 1:200–1:1,600). All other polyclonal sera tested showed only the diffuse pattern of fluorescence whatever the serum dilution used; this was true for *P. falciparum* infections acquired accidentally by blood transfusion, heterologous human infections with *P. vivax*, *P. ovale*, *P. malariae* or *P. cynomolgi*, and experimental animal infections with *P. berghei*, *P. gallinaceum*, or *P. cynomolgi*. Fluorescent antibody titers on PFEEF were generally 1–4 dilutions lower than on blood stages. No age-dependent pattern of fluorescence titers was found in 30 sera from individuals ranging in age from 2–78 years living in a malaria-endemic area. Twenty-six monoclonal antibodies directed to *P. falciparum* blood stages which reacted at high titers with rings, schizonts, merozoites, and gametocytes did not react with PFEEF antigen even when using the undiluted ascitic fluid. Results were also negative when using a monoclonal antibody directed to *P. falciparum* sporozoite surfaces and one to *P. flaciparum* gametes. The results indicate that, in addition to antigens of broad specificity which are shared with blood stages and other malaria species, the hepatic stage of *P. falciparum* exhibits stage- and species-specific antigens which are located at the periphery of the schizont.

Research on human malaria parasites has been focused for many years on the blood stages, and only recently on the sporogonic stages. Tissue forms were the latest to be recognized,[1] and to date little is known about them except for some aspects of their morphology. For example, the hypothetical continuous cycle once believed to be the source of relapses is now challenged by the recent discovery of the hypnozoite forms of *Plasmodium cynomolgi* and *P. vivax*.[2]

We have shown that exoerythrocytic forms of *P. falciparum* (PFEEF) can develop in a South American monkey which is not receptive to blood stages.[3] The large number of PFEEF recovered from the liver permitted a preliminary study of their ultrastructure,[4] and an analysis of their antigens and antigenic relationships to other stages which we report here.

Accepted 13 October 1983.

MATERIALS AND METHODS

Antigens and fluorescent test

Exoerythrocytic forms of *P. falciparum* were obtained by inoculation of 1×10^6 sporozoites of a Gabonese strain into the monkey *Cebus apella*, as described previously.[3] Liver fragments collected on day 5 following infection were either fixed in Carnoy's solution and embedded in paraffin using ordinary methods, or kept in liquid nitrogen.

Most of the fluorescence studies were performed according to the method of Krotoski et al.,[5] using 5-μm sections of Carnoy-fixed frag-

ments which were deparaffinized in xylene and rehydrated in phosphate buffer before use. Sections were incubated with serum dilutions for 30 min at 37°C in a moist chamber, washed, and incubated with fluorescein isothiocyanate antiglobulin (anti-human and anti-mouse Ig from the Pasteur Institute diluted 1:100 in a 1:2,000 solution of Evans blue dye; anti-monkey and anti-chicken Ig from Nordic Laboratories diluted 1:20). Following washing and mounting with a coverslip in glycerine-phosphate buffer, at least four schizonts were observed per dilution tested under 600× and 1,200× magnification using a Leitz microscope equipped with epi-fluorescence. Fluorescent bodies were located by stage coordinates and identified after restaining by the Giemsa-colophonium technique (Fig. 1). It appeared later that artefacts were rare, and that exoerythrocytic forms could be easily identified by light microscopy simultaneously with fluorescence without using Giemsa (see Figs. 4 and 6). Titers are defined as the highest dilution showing fluorescence of PFEEF.

Some sera were retested by the fluorescence antibody test using as antigen 5-μm cryosections from liquid nitrogen-frozen fragments warmed to room temperature and washed before use.

In addition, all sera were simultaneously titrated on blood-stage antigen using thin smears of *P. falciparum* intraerythrocytic schizonts from asynchronous cultures of strain FCR_3/FMG.

Sera

We tested a variety of sera from homologous and heterologous infections in humans and experimental infections in animals, and monoclonal antibodies against *P. falciparum* asexual and sexual blood stages, and sporozoites. All sera were filtered through 0.22-μm membranes, and 2-fold dilutions ranging from 1:50–1:1600 were tested initially. Sera negative at 1:50 were again tested using serum undiluted or diluted up to 1:10.

The anti-*P. falciparum* sera and antibodies used can be classified into six groups as follows:

1. Fourteen sera from African adults continually exposed to malaria in *P. falciparum*-endemic areas.

2. Nine sera from French citizens with primary attacks of falciparum malaria who were infected during brief visits to an endemic area.

3. Four sera from French patients infected accidentally by transfusion with *P. falciparum*-contaminated blood.

4. Twenty-six monoclonal antibodies directed against asexual blood stages of *P. falciparum*: 11 of these were obtained by fusing splenocytes from mice immunized with a Senegalese strain with P3-X 63 Ag 8 myeloma cells as described by Perrin et al.;[15] 15 others were raised against UPA/PLF 3 and FCPS 25 strains as described previously.[7] Supernatants from batch cultures and ascitic fluids were tested.

5. One monoclonal antibody (ascitic fluid) directed to *P. falciparum* circumsporozoite antigen (a gift from E. Nardin).[8]

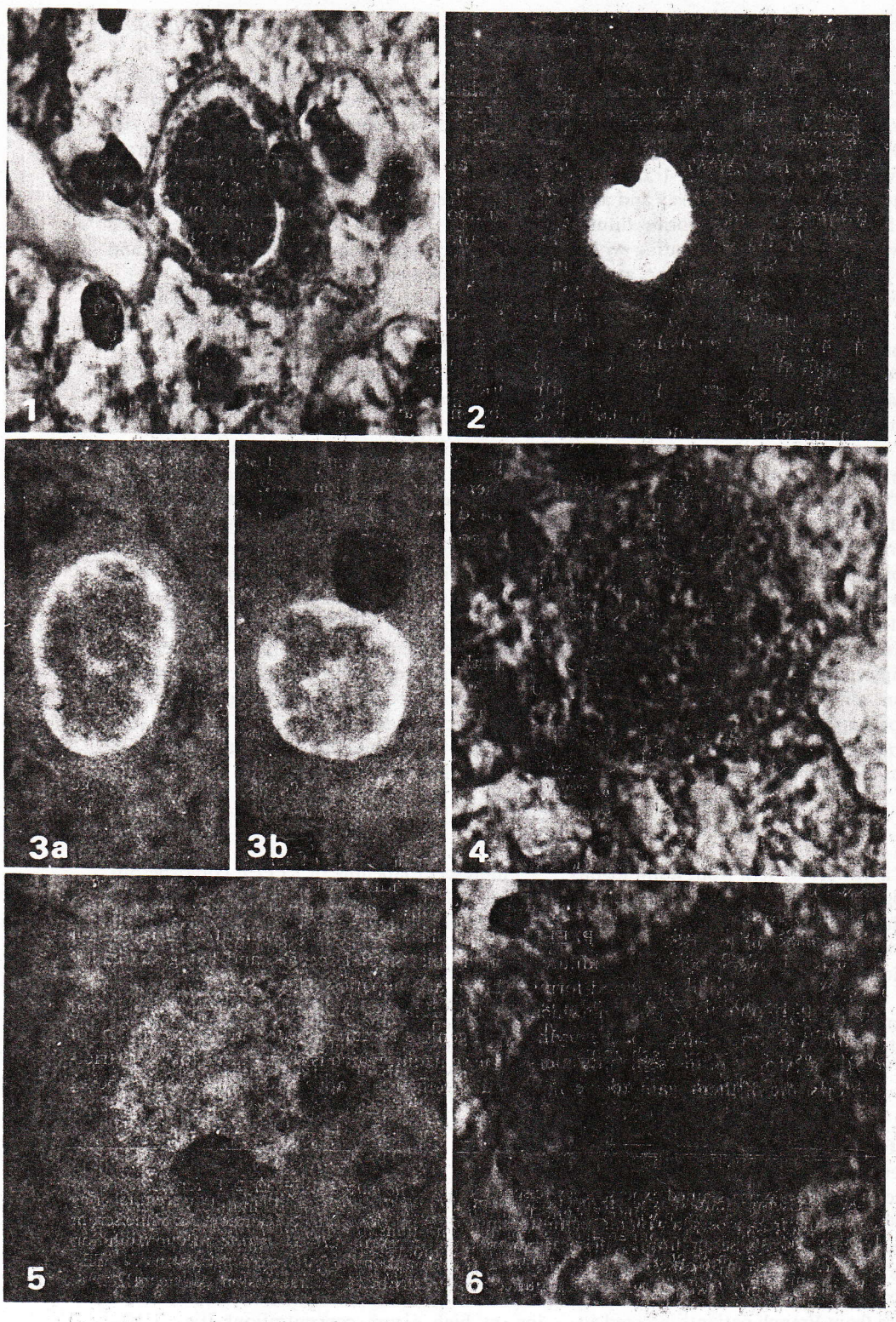
6. One monoclonal antibody (batch culture supernatant) reactive with *P. falciparum* gametes (L. Perrin, unpublished results).

In addition, we also titrated 30 sera from subjects ranging in age from 2–15 years and more than 50 years old living in a village of a rural area of Upper-Volta (Donsé Village).

Sera containing heterologous antibodies were obtained from individuals with primary attacks by *P. vivax, P. ovale,* or *P. malariae;* from two humans infected with *P. cynomolgi*[9] (collected after recovery); from Swiss albino mice experimentally infected with *P. berghei;* from rhesus monkeys infected with *P. cynomolgi bastianellii,* and from chickens infected with *P. gallinaceum.* In each of these heterologous infections, malaria was transmitted by mosquitoes.

RESULTS

Fluorescent labelling of PFEEF could be obtained with all polyclonal sera tested, but differences in titers and localization of fluorescence were observed. At high serum concentrations, the schizonts stained intensely and stood out brightly from the rest of the liver tissue (Fig. 2). At lower serum concentrations, two types of fluorescence were observed, diffuse and peripheral. Diffuse fluorescence (Fig. 5) appeared as a more or less homogeneous pattern of fluorescence all over the surface of the sectioned schizont, which decreased with dilutions of sera but remained diffuse up to the highest positive dilution. Peripheral fluorescence (Fig. 3) appeared as a ring in which the external limit was smooth and seemed to correspond to the membrane of the schizont, and the internal limit was irregular. With *P. falciparum* antisera showing this type of staining, at high serum concentrations (i.e., 1:10–


1
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3a
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1:100) fluorescence was diffuse with peripheral reinforcement, while at all higher dilutions (i.e., 1:200–1:1,600) fluorescence was only peripheral.

Results obtained with each type of serum tested are summarized in Table 1. All sera from heterologous infections in humans and in animals showed only the diffuse pattern of fluorescence. Peripheral staining was observed only with sera from persons infected with the homologous parasite, *P. falciparum,* and solely from those infections naturally acquired through mosquito bites. None of four sera from persons with *P. falciparum* infections induced by accidental intravenous inoculation of blood stages (transfusion malaria) showed peripheral fluorescence. In contrast, each of the 23 sera from persons with mosquito-transmitted falciparum malaria had antibodies binding to the periphery of the schizont.

No significant difference in titer or localization of fluorescence was found between the group of African immune adults and the group with primary attacks of malaria. Similarly, no direct relationship seemed to exist between antibody titers on PFEEF and age. In the subjects from the village of Upper-Volta mean titers were as follows: 2- to 5-year age group, 344 ± 247; 5–10 years, 253 ± 255; 10–15 years, 214 ± 232; >50 years, 515 ± 340.

Whatever the type of serum tested, no staining of the host cell was seen, a striking difference from results obtained with malaria blood stages. Simultaneous titration of sera on infected red blood cells revealed that all were positive at titers generally 1–4 dilutions higher than on PFEEF (data not shown).

Surprisingly, all monoclonal antibodies raised against blood stages did not react with PFEEF antigen. This was true even when using undiluted ascitic fluids, with which titers on blood forms were in the range of 1:5,000–1:100,000, and which were able to bind to various stages of the erythrocytic cycle (rings, trophozoites, schizonts, merozoites and, for some of them, gametocytes).

Negative results were also found when using the undiluted ascitic fluid from one anti-sporozoite hybridoma, which was highly positive on glutaraldehyde-fixed *P. falciparum* sporozoites of the strain used to infect the monkey. No labeling occured when culture supernatant from one anti-gamete monoclonal antibody was used.

Since the absence of reaction with monoclonal antibodies could possibly be attributed to an alteration of antigens following Carnoy's fixation and further treatments, the tests were repeated with frozen sections from liquid nitrogen-preserved liver tissue. The use of this type of antigen resulted in considerable nonspecific background staining, and in staining of the nuclei of hepatocytes with malarial sera due to their content of anti-nuclear antibodies.[10] However, no fluorescence of PFEEF occurred with the latter antigenic preparation with any anti-sporozoite and anti-merozoite monoclonal antibodies tested undiluted to 1:500, while all polyclonal sera were unequivocally positive.

DISCUSSION

Stage-specific antigens have been identified in malaria sporozoites,[8] merozoites,[11] and gametes,[12] and were found to be located on the surface of these stages. The present results confirm that exoerythrocytic forms (EEF) also exhibit stage-specific antigens, and that antigenic changes occur between each developmental stage of the cycle of *P. falciparum,* particularly between the asexual liver and blood stages.

The great variety of sera showing diffuse fluorescence indicates that some internal antigens revealed by sectioning PFEEF are of broad specificity, having antigenic similarities with those of many plasmodial species, and being shared by blood and hepatic stages.

Our data also show the existence of an original antigenic structure, located at the periphery of the schizont, which is remarkable for its restricted specificity. It appears species-specific, being

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FIGURES 1–6. **1.** Giemsa-stained exoerythrocytic schizont of *P. falciparum* (PFEEF); **2.** Fluorescent labeling of PFEEF with serum from a person with mosquito-transmitted *P. falciparum* at 1:50 dilution; **3a.** Same serum as in **2**, diluted 1:1,600; **3b.** Serum from another case of mosquito-transmitted *P. falciparum* infection, dilution 1:800); **4.** Same schizont as in **3b** observed under visible light without staining; **5.** Fluorescent pattern of PFEEF obtained with serum from *P. falciparum* malaria acquired accidentally by blood transfusion, dilution 1:200; **6.** Same schizont as in **5** under visible light.

TABLE 1

Location of fluorescence and mean titers on P. falciparum *exoerythrocytic schizonts*

Sera	No. tested	Fluorescence*	
		Diffuse	Peripheral
Normal human serum	2	0	0
P. falciparum antisera			
Sporozoite-induced infections			
Multiple attacks	14	++	+++
Primary attack	9	++	+++
Merozoite-induced infections (transfusion malaria)	4	++	0
Anti-blood stage monoclonal antibodies	26	0	0
Anti-gamete monoclonal antibody	1	0	0
Anti-sporozoite monoclonal antibody	1	0	0
Human sera from sporozoite-induced infections with:			
P. vivax	6	++	0
P. ovale	5	++	0
P. malariae	3	++	0
P. cynomolgi	2	++	0
Animal sera			
P. cynomolgi in rhesus monkeys	2	+	0
P. berghei in Swiss albino mice	2	+	0
P. gallinaceum in chickens	2	+	0

* 0, no fluorescence with serum undiluted up to 1:10; −, titer ≥1:100; ++, 1:100–1:200; +++, 1:400–1:1,600.

revealed only by sera from mosquito-acquired *P. falciparum* infections. The fact that sera from subjects with transfusion malaria did not react demonstrates that this antigenic structure belongs only to the pre-erythrocytic stages. Furthermore, its absence of cross-reactivity with the circumsporozoite antigen suggests that it could be strictly specific to the liver stage. This hypothesis is supported by previous studies of animal malaria, which showed that polyclonal anti-sporozoite serum stained young but not late EEF,[13,14] and that such serum had no effect on the development of established EEF.[15]

The cross reactivity of the internal antigens with antibodies to heterologous species is in agreement with the previous study of Krotoski et al.,[16] who used *P. knowlesi* EEF and *P. cynomolgi* antiserum, but not with that of El Nahal.[17] In that study no reactivity was found between either *P. cynomolgi* EEF and other simian malaria antisera, or *P. malariae* EEF and *P. falciparum* antisera. This discrepancy might be due either to differences in maturation of the EEF, or differences in sensitivity of the fluorescent assays used.

An interesting and puzzling finding was the failure of anti-blood stage monoclonal antibodies to stain PFEEF. Many of these were found to react with air-dried merozoite preparations, and to precipitate radio-labeled antigens extracted from merozoites (unpublished results). One possibility is that the so-called merozoite released from EE schizonts may differ antigenically from the merozoite released by asexual schizonts in the blood. Indeed, both merozoites also differ in size and shape.[18] Another possibility is that the merozoite-specific antigens only appear in the late phases of maturation and segmentation of the EEF. Therefore, they would not be present in our PFEEF which, although they were taken on the 5th day of infection, were not fully matured.

Since none of the anti-blood stage monoclonal antibodies were directed to those antigens common to blood and hepatic stages, it may indicate that mice immunized by *P. falciparum* blood stages respond to some, but not all, of the erythrocytic antigens.

The peripheral fluorescent structure could not be precisely identified by the assay used in our study. However, its regular external shape and irregular inner limit may correspond to the vacuolar system seen with the electron microscope, which contains granules some of which are present in the intermembraneous space of the parasitophorous vacuole.[4]

Within the limitations that the schizonts studied were obtained in an unusual host in which antigens expressed may differ from those expressed in man, the present study shows that hepatic stage *P. falciparum* antigens exhibit inter-stage and inter-species cross-reactivity but that, as in the sporozoites, merozoites and gametes, the adaptation of the parasite to this phase of its life cycle has led to the expression of antigens with a highly restricted specificity.

ACKNOWLEDGMENTS

We want to express our thanks to Elisabeth Nardin-Higgins and Ruth Nussenzweig for supplying the anti-sporozoite monoclonal antibody used in this study.

This work was supported by grants from DGRST/MRI to the CHU Pitié-Salpêtrière, and from UNDP/World Bank/WHO Tropical Diseases Research Programme to the Muséum d'Histoire Naturelle and to the WHO/IRTC of the Geneva Blood Center.

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