

ABSENCE OF SPOROZOITE-NEUTRALIZING ACTIVITY OF ANTIBODIES RECOGNIZING THE *PLASMODIUM FALCIPARUM* SEXUAL STAGE ANTIGEN Pfs16

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Summary :

Pfs16 was isolated independently by two groups of researchers either by screening cDNA and genomic expression libraries with monoclonal antibodies raised against purified gametocytes of *Plasmodium falciparum*, or by a subtractive cloning strategy. Expression of this antigen has been found unambiguously in gametocytes by both teams but in sporozoites by only one group. Moreover, the latter group reported that antibodies raised against recombinant Pfs16 proteins could prevent sporozoite penetration into human hepatoma cells and human hepatocytes. In contrast we have found that monoclonal and polyclonal antibodies specifically recognizing Pfs16 in gametocytes do not react with sporozoites. Moreover, they were shown to have no inhibitory activity against *P. falciparum* sporozoite penetration in human hepatocytes.

KEY WORDS : *Plasmodium falciparum*, malaria, sporozoite, gametocyte.

MOTS CLÉS : *Plasmodium falciparum*, malaria, sporozoite, gametocyte.

Résumé : ABSENCE D'ACTIVITÉ NEUTRALISANT LE SPOROZOÏTE DES ANTICORPS CONTRE Pfs16, UN ANTIGÈNE GAMÉTOCYTAIRE DE *PLASMODIUM FALCIPARUM*

Pfs16 a été isolé de façon indépendante par deux groupes de chercheurs soit par identification de clones dans des banques d'expression d'ADN complémentaires ou génomiques à l'aide d'anticorps monoclonaux obtenus d'animaux immunisés avec des gamétoytes de *Plasmodium falciparum*, ou soit par une stratégie de clonage par soustraction. L'expression de cet antigène a été démontré de façon nette sur le gamétoyte par les deux groupes, mais seulement sur le sporozoïte par un des deux groupes. De plus, ce dernier a décrit que des anticorps obtenus de souris immunisées avec des protéines Pfs16 recombinantes peuvent prévenir la pénétration des sporozoïtes dans des cellules d'hépatomes et d'hépatocytes humains. Au contraire, nous avons observé que des anticorps monoclonaux et polyclonaux qui reconnaissent de façon spécifique Pfs16 sur le gamétoyte ne réagissent pas contre le sporozoïte. De plus, ces anticorps n'inhibent pas la pénétration de sporozoïtes de *P. falciparum* dans les hépatocytes humains.

INTRODUCTION

Malaria infection is initiated by the bite of an infective mosquito which injects sporozoites into the host's circulation. Sporozoites rapidly invade hepatocytes where they form schizonts that give rise to thousands of merozoites. Vaccine strategies based on the blocking of sporozoite invasion have been developed and rely on identification of target antigens on the surface of the sporozoite (Nussenzweig & Nussenzweig, 1989). Despite extensive research, few antigens have been identified. Antibodies against the circumsporozoite protein (CSP) (Dame

et al., 1984; Enea *et al.*, 1984) and another sporozoite surface protein, SSP2/TRAP, have been shown to inhibit sporozoite penetration into human hepatocytes (Mazier *et al.*, 1986; Rogers *et al.*, 1992).

Recently, Pfs16, a protein first described as being expressed in gametocytes (Bruce *et al.*, 1990; Moelans *et al.*, 1991), the sexual stage of the parasite, was proposed as being expressed also on the sporozoite surface. Polyclonal antibodies raised against a peptide and a fusion protein of Pfs16 recognized a band with an apparent molecular mass of 16 kDa and gave positive staining by immunofluorescence and immunoelectron microscopy on *P. falciparum* sporozoites (Moelans *et al.*, 1991, 1995).

If antibodies to Pfs16 induced by immunization with recombinant proteins have the capacity to inhibit sporozoite penetration and block transmission to the mosquito, this molecule would be an important vaccine candidate. Thus, we undertook to repeat the studies using monoclonal antibodies and polyclonal antibodies recognizing Pfs16 (Baker *et al.*, 1994; Bruce *et al.*, 1990, 1994).

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MATERIALS AND METHODS

PARASITES

P. falciparum (strain NF54) cultures were grown in a semi-automated continuous flow apparatus in RPMI-1640 (Sigma, Saint-Quentin-Falavier, France) supplemented with 25 mM HEPES buffer and 10 % human serum (A + and supplied with filter-sterilised gas (90 % N₂, 5 % O₂ and 5 % CO₂). Gametocytes were harvested after 14-16 days by percoll gradient centrifugation as described previously (Harte, Rogers & Targett, 1985). Sporozoites were obtained from salivary glands of *Anopheles stephensi* mosquitoes infected after feeding through an artificial membrane on gametocytes from cultures of the NF54 strain.

ANTIBODIES

Three monoclonal antibodies, 2G7, 43D6H12 and 93A3A2 recognizing different epitopes of Pfs16 (Baker *et al.*, submitted) were used: mAb 2G7 recognizes an epitope at the C terminus of the Pfs16 molecule contained in the peptide KDKDKDNTDE corresponding to amino-acids 131-140 of the protein; mAb 43D6H12 recognizes an epitope in the N-terminal part contained in the peptide AVGPN corresponding to amino-acids 66-70 of the protein; mAb 93A3A2 recognizes an epitope located in the N-terminal of the protein (amino-acids 1-76) but different from the epitope recognized by the mAb 43D6H12. MAb 93A3A2 was used as ascites or after purification on a protein A-sepharose column and displayed IFAT titer < 50,000. Mabs 2G7, 43D6H12 were shown to recognize a unique band of 16 kDa in western blot studies (Bruce *et al.*, 1994) and to localize this antigen as an internal protein in gametocytes (Bruce *et al.*, 1994; Baker *et al.*, 1994). A rabbit polyclonal serum, Eowyn, obtained after five injections subcutaneously of 25 µg recombinant Pfs16 with 25 µg of saponin as an adjuvant in a New Zealand White Rabbit, was also tested. The protein used for immunization represents the entire coding region of the Pfs16 gene and was produced in *E. coli* as previously described (Baker *et al.*, 1994). In Western blots, this serum reacted with both native and reduced forms of the Pfs16. A strong reaction with gametocytes was observed with acetone-fixed parasites in IFAT (titer < 50,000), but a relatively weak reaction occurred with rounded up gametocytes undergoing gametogenesis. This serum also allowed to identify the subcellular localization of Pfs16 in gametocytes (Baker *et al.*, 1994). Monoclonal antibodies, E9 (Boulanger, Matile & Betschart, 1988) and CT1 (Del Giudice *et al.*, 1991), specific for the repeat of the circumsporozoite protein (CSP) were also used and displayed IFAT titer of 51,200.

IMMUNOFLUORESCENCE ASSAY (IFA)

Immunofluorescence assay was performed with gametocytes and three different preparations of *P. falciparum* sporozoites.

i) « Wet » sporozoites. Live sporozoites were handled as previously described to detect membrane staining (Rénia *et al.*, 1988). Briefly, freshly isolated *P. falciparum* sporozoites were deposited on glass slides coated with poly-L-Lysine and kept alive at 4 °C for 24 hours in a moist chamber to allow attachment.

ii) Air-dried sporozoites. Sporozoites were deposited on glass slides, air-dried and fixed with methanol.

iii) Live sporozoites. Aliquots of freshly isolated sporozoites were incubated for 30 minutes at 4 °C in a Eppendorf tubes with the different antibodies. They were washed by centrifugation at 15,000 rpm for 10 minutes and then incubated with the secondary fluoresceinated anti-mouse Ig. After washing, sporozoites samples were deposited on U-shaped slides (Paul Block, Strasbourg, France) which were covered with glass cover-slips (CML, Nemours, France) and observed under a fluorescence microscope (Olympus, Paris, France).

iv) Air-dried gametocytes. Percoll-purified gametocytes were pipetted onto multiwell slides, air dried, acetone fixed and stored at - 20 °C.

25 µl of serum in a 1:100 dilution in PBS were added to each well of antigen-coated slides. They were incubated in a moist chamber at room temperature for 30 minutes and then washed 4 times in PBS. 25 µl of a 1:100 dilution (in PBS) of FITC-conjugated anti-mouse Ig (Nordic, Tilburg, Netherlands) were added to each well and incubated at room temperature for a further 30 minutes. The slides were washed as above and mounted with buffered glycerol, and viewed with a Leitz UV microscope.

IMMUNOELECTRON MICROSCOPY

Sporozoites and Percoll-purified gametocytes which had been stimulated to undergo gametocytogenesis were fixed for 30 minutes with 2 % paraformaldehyde and 0.1 % glutaraldehyde in PBS, dehydrated in ethanol and embedded in LR White (London Resin Co.). Ultra-thin sections were collected on piliform-coated nickel grids and floated on PBS/1 % BSA/0.01 % Tween 20 (PBS/BSA/T). The grids were then floated on various dilutions of the primary antibody overnight at 4 °C and then 1-2 hours at room temperature (RT). This was followed by several washes with PBS/BSA/T and incubation with secondary antibody; goat anti-mouse or rabbit IgG-5 nm gold conjugate (Bio-cell research Labs, London) at RT for 1-2 hours. After washing with PBS/BSA/T, PBS/T and ultrapure water for several min, the sections were silver enhanced using an IntenSE M silver

enhancement kit (Amersham International, Inc., London) according to the manufacturer's instructions. The sections were stained with a saturated uranyl acetate solution in 30 % methanol followed by Reynolds lead citrate and then observed with a JEOL 1200EX electron microscope at 80 kV.

WESTERN BLOT ANALYSIS

Samples, containing 75×10^3 sporozoites (obtained from infected mosquitoes and freeze-thawed three times), were boiled for 15 minutes in 25 μ l of sample buffer (62.5 mM Tris/HCl, 2 % SDS, 10 % glycerol, 10 % 2-mercaptoethanol, 0.003 % Bromophenol blue, pH 7.2). Samples were subjected to SDS-PAGE on 5-15 % gradient gels. The samples were then transferred to nitrocellulose by electroblotting (Towbin, Staehelin & Gordon, 1979). Western blots were washed with PBS containing 0.1 % Tween-20 and blocked in $1 \times$ PBS with 5 % dried milk powder/0.001 % Tween-20 (Sigma) for 2 hours at 4 °C before incubation with antibodies diluted in $1 \times$ PBS with 5 % dried milk powder/0.01 sodium azide. Antibodies used were: mAbs CT1 and E9 specific for the repeat region of the CSP, mAbs 2G7 and 93A3A2 specific for Pfs16, all at a 1:100 dilution of mouse ascitic fluid and rabbit anti-Pfs16 serum Eowyn, used at a 1:100 dilution. Blots were incubated for 3 hours or overnight. Visualization of antigens was performed as previously described (Del Giudice *et al.*, 1991).

INHIBITION OF PENETRATION OF *P. FALCIPARUM* SPOROZOITES

Human hepatocytes were isolated by collagenase perfusion of liver biopsies as previously described (Mazier *et al.*, 1986). Cells were maintained for 24 hours before sporozoite inoculation at a concentration of 10^5 cells per well in Labtek plastic cultures slides (Miles Lab., Elkhart, IN, USA) in William's medium (Gibco, Edinburgh, Scotland) containing 10 % fetal calf serum (Gibco) and Penicillin-Streptomycin (stock solution, 100 \times , Gibco). The sporozoites were obtained by dissection of the salivary glands of *Anopheles stephensi* infected with *P. falciparum* strain NF54. Inhibition assays were performed by adding 4×10^4 sporozoites with purified mAbs 2G7, 93A3A2 or 43D6H12 at a final concentration of 100 μ g/ml, or heat inactivated rabbit sera (Eowyn or normal serum) at a final dilution of 1:10. A heat inactivated rabbit serum against a peptide (NANP)₄₀ repeat region of CSP (IFAT titer on sporozoite: 102,400) was used at the same final dilution as a positive control. Fresh medium without antibodies was added 3 hours later. The presence and location of the parasite in the hepatocytes were determined 24 hours after sporozoite inoculation with a double staining

assay using anti-CSP mAb labeled with peroxidase followed by antibody labeled with fluorescein as previously described (Rénia *et al.*, 1988). In order to rule out any non-specific effect, antibodies were also tested in inhibition assays using mouse hepatocytes and sporozoites of the rodent malaria parasite, *P. yoelii*. They displayed no inhibitory activity in this system.

RESULTS

REACTIVITY OF ANTI-PFS16 ANTIBODIES

In an immunofluorescence assay, all antibodies displayed strong staining on gametocytes (data not shown) as previously described (Baker *et al.*, 1994; Bruce *et al.*, 1994). When assayed by immunofluorescence on methanol-fixed, « wet sporozoites » or live sporozoites of *Plasmodium falciparum* of the NF54 strain, no staining was observed with these antibodies. Using the same technique on the same batch of sporozoites, staining was observed when E9, a monoclonal antibody specific for the repeat region of CSP (Boulanger, Matile & Betschart, 1988), was used. The same results were obtained with sporozoites of the Ro strain of *P. falciparum* (data not shown).

Western blot analysis and immunoelectron microscopy were further used to try to detect Pfs16 in sporozoites. No band of 16 kDa was detected by western blotting and no reactivity could be demonstrated on sporozoites by immunoelectron microscopy using the anti-Pfs16 monoclonal antibodies and one rabbit polyclonal antibody (Eowyn). In the contrary, these mAbs were able to detect a band of 16 kDa in gametocytes preparation used in western blotting as previously reported (Bruce *et al.*, 1994; Baker *et al.*, 1994) (data not shown). Anti-CSP mAb CT1 on the other hand revealed by western blotting the presence of two bands corresponding to the expected m.w. of the CSP (Del Giudice *et al.*, 1991) (data not shown).

ABSENCE OF SPOROZOITES-NEUTRALIZING ACTIVITY OF ANTIBODIES TO PFS16

The ability of these different antibodies to inhibit sporozoite penetration was evaluated since we have shown previously that some antibodies against the repeat region of the CSP could have an inhibitory effect despite displaying no reactivity to sporozoite as determined by indirect immunofluorescence or Western blot (Del Giudice *et al.*, 1991). Anti-Pfs16 monoclonal antibodies and rabbit sera had no effect on *P. falciparum* sporozoite penetration (Table I). Rabbit serum against the repetitive region of the CSP inhibited sporozoite penetration (Table I) as previously described (Mazier *et al.* 1986).

Antibodies	Origin	gamIFA*	spzIFA**	% Inhibition of sporozoite penetration***
93A3A2	mouse ^a	+	-	9 ± 7
43D6H12	mouse	+	-	0
2G7	mouse	+	-	0
Ewoyn	rabbit	+	-	0
Normal serum	rabbit	-	-	0
Anti-(NANP) ₄₀	rabbit	-	+	67,5 ± 7 ^b

* Indirect immunofluorescence on acetone-fixed gametocytes.

** Indirect immunofluorescence on methanol-fixed, « wet » or live sporozoites.

*** Sporozoites were added to hepatocytes with or without the antibodies and cultures were stopped 24 hours later. Sporozoites were revealed by the double staining assay and only sporozoites which had penetrated were counted. Results are expressed as mean ± SE of reduction of sporozoite penetration as compared to control wells and represent results from two separate experiments.

^a Antibodies of mouse origin are monoclonal antibodies.

^b Significantly lower than the value for cultures with normal rabbit serum (P < 0.01; student t-test).

Table I. — Assay for inhibition of *P. falciparum* sporozoite penetration in hepatocytes *in vitro* by antibodies against Pfs16.

DISCUSSION

We conclude from these experiments that anti-Pfs16 antibodies used in this study, (either polyclonal or monoclonal) did not display reactivity against sporozoites and do not inhibit parasite penetration into human hepatocytes. Our results differ from those of Moelans *et al.* (1991, 1995). They found that antibodies raised against peptides and recombinant proteins of Pfs16 recognized sporozoites in Western blots, by immunofluorescence and by immunoelectron microscopy. There was no indication in these papers of the concentration of antibodies used in these assays and the possibility of cross-reactivity with other sporozoite antigens need to be considered. Another possibility is that Pfs16 is expressed at very low level undetectable in our hands. Moelans *et al.* (1995) also reported that their antibodies to Pfs16 could prevent sporozoite penetration into human hepatoma cells and primary human hepatocytes. Results concerning the use of hepatoma cells should be interpreted with caution since this study and a previous report (Hollingdale *et al.*, 1990) showed that antibody neutralizing activity can differ dramatically if hepatocytes are used instead of hepatoma cells. The sporozoite invasion inhibitory assay (Hollingdale *et al.*, 1984) used by Moelans *et al.* (1995) is also less stringent than the double staining assay used in this study (Rénia *et al.*, 1988) to ascertain whether sporozoites have penetrated the hepatocytes. There is also a need to ensure that the effects on sporozoite penetration and development in hepatocytes are not due to non-spe-

cific factors such as C-reactive protein, 1-antitrypsine, haptoglobuline, and α 2-macroglobulin (Nüssler *et al.*, 1991, Pied *et al.*, 1989, 1995) which could be present in serum. One method, used routinely in our laboratory to do this, is to study the inhibitory capacity of mouse and rabbit sera in a heterologous system (e.g. *Plasmodium yoelii* sporozoites and primary cultures of mouse hepatocytes).

In conclusion, further studies are needed to establish whether Pfs16 induces a specific anti-sporozoite immunity.

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