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Induction of *Plasmodium falciparum* sporozoite-neutralizing antibodies upon vaccination with recombinant Pfs16 vaccinia virus and/or recombinant Pfs16 protein produced in yeast

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Abstract

Pfs16 is a sexual stage/sporozoite-specific antigen of *Plasmodium falciparum* and is a potential candidate for a sporozoite-neutralizing vaccine. To obtain more information on the function of Pfs16 and to investigate its role during transmission and hepatocyte invasion, immunization experiments were performed with both a Pfs16-specific recombinant vaccinia virus and virus-like particles produced in yeast composed of the hepatitis B surface antigen (HBsAg) and antigen Pfs16 fused to HBsAg. Upon transformation of yeast cells, harbouring a genomic copy of the HBsAg gene, with a plasmid carrying the fusion gene Pfs16-HBsAg (Pfs16-S) virus-like hybrid particles composed of HBsAg and Pfs16-S were formed of a size similar to those present in human sera after infection with the hepatitis B virus. Cells infected with recombinant Pfs16 vaccinia virus synthesized a polypeptide of approx. 16 kDa that reacted with a Pfs16-specific polyclonal antibody. Animals vaccinated with the yeast hybrid particles and/or recombinant vaccinia virus both produced Pfs16-specific

Abbreviations: CS protein, circumsporozoite protein; GWB, gametocyte Western blot analysis; ELISA, Enzyme-linked immunosorbent assay; HBsAg, hepatitis B surface antigen; IFA, indirect immunofluorescence assay; ISI, inhibition of sporozoite invasion; Pfs16-S, Pfs16-HBsAg fusion protein; Pfs16-S,S, virus-like particles composed of HBsAg and Pfs16-S; SIFA, suspension indirect immunofluorescence assay; SWB, sporozoite Western blot analysis; TB assay, transmission-blocking assay.

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² Note: This publication is dedicated to the late John Schoenmakers.

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antibodies. These antibodies showed no transmission-blocking activity, but they efficiently diminished or abolished in vitro invasion of sporozoites into human hepatoma cells (HepG2-A16) and primary human hepatocytes.

Keywords: *Plasmodium falciparum*; Pfs16; Recombinant vaccinia virus; Heterologous production; Yeast; Sporozoite-neutralizing vaccine

1. Introduction

Sporozoites are the infective stage of the malaria parasite and are found in the salivary glands of female *Anopheles* mosquitoes. During feeding, mosquitoes inject sporozoites into the host's circulation which rapidly invade hepatocytes and develop into thousands of merozoites. After rupture of hepatocytes, these merozoites initiate repeated cycles of erythrocyte infection associated with clinical disease. Irradiated sporozoites elicit protective immunity in rodents, monkeys and humans and led to the identification of the circumsporozoite (CS) protein and the sporozoite surface 2 protein (SSP2) [1–5]. There is now compelling evidence indicating that the immunity induced by irradiated sporozoites is mediated by anti-sporozoite antibodies, as well as CD4+ and CD8+ cells that recognize sporozoite-infected hepatocytes [6]. CS protein has been the focus of most efforts to develop a pre-erythrocytic stage malaria vaccine. However, despite many efforts, thus far only a few volunteers have been protected with recombinant or synthetic CS vaccines [7,8].

Recently, we reported the presence of the highly conserved 16-kDa protein Pfs16 on the surface of both sexual-stage parasites and sporozoites [9,10]. Rabbit sera raised against a synthetic peptide and against recombinant fusion proteins produced in *Escherichia coli* recognize a 16-kDa antigen in protein extracts of gametocytes, macrogametes and sporozoites by Western blot analysis [9]. By one- and two-dimensional Western blot analysis using Pfs16 specific monoclonal antibodies it was demonstrated that these 16-kDa proteins produced by gametocytes and sporozoites are identical (unpublished data). By immunoelectron microscopy, Pfs16 was localized on the surface of sporozoites and in the parasitophorous vacuole membrane of gametocytes [9,11,12].

We further investigated whether Pfs16 is a candidate malaria vaccine antigen that might elicit protec-

tive antibodies that could neutralize sporozoite infectivity or block transmission. To begin development of suitable Pfs16 vaccines, we report in this study the expression of Pfs16 or Pfs16 fragments, in yeast and mammalian cells. Yeast provides a simple and inexpensive means for recombinant protein production [13]. Very recently, the RTS,S vaccine produced in yeast, containing the hepatitis B virus envelope protein as a carrier for B-cell and T-cell epitopes of the central repeat region and the C-terminal region of the CS protein, has proven to be safe and immunogenic during a clinical study conducted in Thailand [14]. Live attenuated viruses, such as vaccinia virus are attractive vectors for vaccine development because they are easily administered, inexpensive to produce and stress-resistant during transport. Vaccinia virus has been used for the production of a large number of antigens from pathogenic organisms, and in several cases vaccination with these recombinant viruses conferred immunity to a subsequent challenge [15,16]. Viral vectors stimulate different arms of the immune system, which is valuable for vaccine development [17,18].

Antisera were prepared with both recombinant vaccinia virus and a recombinant Pfs16 fusion protein prepared from yeast. These sera were tested for their capacity to inhibit in vitro sporozoite invasion into human hepatoma cells and hepatocytes, and transmission-blocking activity.

2. Materials and methods

2.1. Construction of recombinant plasmids

2.1.1. Tcd5 / 16-S

Recombinant plasmids were constructed and isolated by standard methods using *E. coli* MM294 (F⁻, endA1, thi, hsdR17, supE44, lambda⁻) as "host" [19]. For the production of Pfs16-specific

fusion proteins in yeast the shuttle vector Tcd5 was used. It contains an expression cassette consisting of the yeast glyceraldehyde-3-phosphate dehydrogenase (TDH3) promoter fused to the gene coding for HBsAg followed by a yeast transcription termination region of the ARG3 gene. The Leu2 gene serves as a selection marker. Both at the 5' and 3' ends of the expression cassette TY1 sequences were inserted [20] to achieve homologous recombination between the expression cassette and TY1 sequences present within the yeast genome. To create fusions between the Pfs16 gene and the gene coding for the hepatitis B surface antigen (HBsAg) a PCR fragment coding for amino acids 20–104 of Pfs16 was cloned into the unique *Sma*I site of the shuttle vector Tcd5 (Fig. 1A). The plasmid containing the Pfs16 sequence was designated Tcd5/16-S. The resulting chimeric gene

encodes a protein (Pfs16-S) of which the deduced amino-acid sequence is shown in Fig. 1B.

The Tcd5/16-S plasmid was digested with *Xho*I and the resulting fragments were separated by agarose-gel electrophoresis according to standard procedures [19]. The fragment Tcd5/16-S-*Xho*I, containing the TY1, Leu2 and the expression cassette sequences, was then isolated by electroelution [19]. Subsequently, *Saccharomyces cerevisiae* strain Y1295 (MAT α , leu2-3, leu2-112), already containing 4–5 copies of the HBsAg gene in its genome, was transformed with the Tcd5/16-S-*Xho*I fragment via the spheroplast transformation method described by Hinnen and co-workers [21]. Stable transformants were selected by selection for leucine auxotrophy. The transformant containing both a copy of the Pfs16-S fusion gene and 4–5 copies of the HBsAg

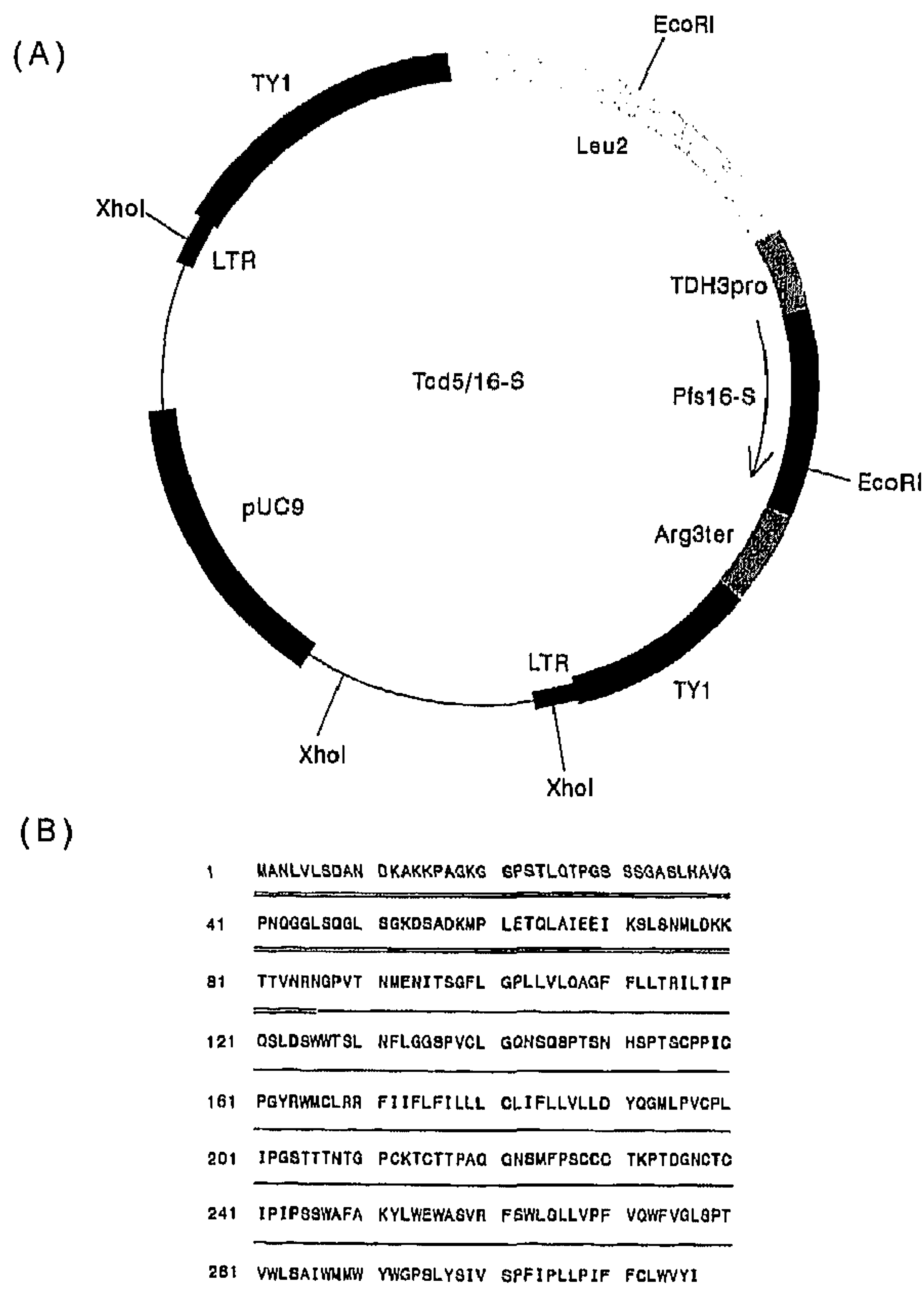


Fig. 1. (A) Schematic representation of plasmid Tcd5/16-S. (B) Amino-acid sequence of the fusion protein Pfs16-S. It consists of the Pfs16 antigen (====) devoid of the signal peptide sequence (amino acids 1–19) and the putative membrane anchor and cytoplasmic region (amino acids 105–157) fused to the hepatitis B surface antigen (—).

gene was called Y1655 and the hybrid particles composed of HBsAg and Pfs16-S (Pfs16-HBsAg fusions) were designated Pfs16-S,S.

2.1.2. Construction of recombinant vaccinia virus transfer vector pSC11/16

Transfer vector pSC11 contains a compound early/late promoter of moderate strength, P_{7.5}, followed by a unique *Sma*I site and flanked by fragments (TKl and TKr) of the vaccinia thymidine kinase (TK) gene [22]. It also contains the late vaccinia virus promoter P₁₁, that is used for the expression of the *E. coli* lacZ reporter gene. The recombinant Pfs16 specific transfer vector pSC11/16 was constructed via insertion of a PCR fragment, encompassing the complete coding information of Pfs16, into the unique *Sma*I site. Recombinants were constructed and propagated by standard methods [19].

For the construction of recombinant viruses according to standard methods vaccinia virus strain WR, a gift of Dr. L. Boryciwicz (University of Cambridge, Cambridge, UK), was used. It was propagated and purified as reported previously [23]. CV-1 cells infected with wild-type vaccinia virus were transfected with calcium phosphate-precipitated plasmid (pSC11/16) DNA [22]. For identification and purification of recombinant viruses TK selection and β -galactosidase screening was used [22]. TK-recombinants producing β -galactosidase were selected by plaque assay on RAT-2 cells (TK⁻) in the presence of 5-bromo-deoxyuridine (BUdR) and X-Gal (60 μ g ml⁻¹). Plaques were visualized after approx. 6 h of incubation at 37°C. After two to three selection procedures, recombinant virus (vSC11/16) was amplified in RAT-2 cells [23]. CV-1 and RAT-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO Laboratories) containing 10% fetal bovine serum (FBS) and for the TK⁻ cells 25 μ g of 5-bromo-deoxyuridine (BUdR) per ml was added to the medium.

2.2. Analysis of the synthesis of recombinant proteins in yeast

Synthesis of the recombinant proteins Pfs16-S and HBsAg was monitored by immunoblot analysis of total extracts using rabbit polyclonal antiserum K37S8 (anti-Pfs16) [9] and mouse monoclonal anti-

body, HBS1 (anti-HBsAg), respectively. Preparation of crude cell extracts and immunoblot analysis was performed as described [24].

2.3. Characterization of yeast particles containing recombinant Pfs16-specific fusion protein

HBsAg synthesized in yeast is accumulated in particles similar in size as the 22-nm particles found in human sera after hepatitis B infection which have a buoyant density of 1.18 g cm⁻³ [25]. To study whether the recombinant Pfs16-S fusion proteins and HBsAg synthesized in yeast Y1655 also accumulate in 22 nm particles, crude extracts of Y1655 yeast cells were subjected to CsCl equilibration centrifugation as described [24]. In short, crude cell extract, equivalent to approx. 15 mg of cell protein, was mixed with 1.5 M CsCl in sodium phosphate buffer (pH 7.4) and centrifuged in a 50 Ti (Beckman) rotor at 45 000 rpm for 72 h at 8°C. After centrifugation 0.5-ml fractions were collected by puncturing the bottom of the tube. Subsequently, 2 μ l of each fraction was analyzed for the presence of HBsAg via the radioimmunoassay AUSRIA II (Abbott Laboratories) and 10 μ l was used for immunoblot analysis to study the presence of the Pfs16-S fusion protein and HBsAg antigen, utilising antisera K37S8 and HBS1, respectively.

2.4. Expression of gene Pfs16 using recombinant vaccinia virus vSC11/16

To study the expression of gene Pfs16 after infection with recombinant vaccinia virus vSC11/16, a confluent RAT-2 cell monolayer was used. 48 h after infection cells were harvested by low-speed centrifugation and the pellet fraction was resuspended in 400 μ l of SDS/sample buffer and heated for 5 min at 95°C. To establish whether a Pfs16 protein of appropriate size was made immunoblot analysis was performed using K37S8 as antiserum.

2.5. Isolation of gametocytes and sporozoites

Isolation of sporozoites was performed using the urografin gradient method as described by van Pelt and co-workers [26]. Gametocytes were isolated as described [9,27].

2.6. Western blot analysis

For Western blot analysis of sporozoite (SWB) and gametocyte (GWB) antigens, samples containing approx. 3×10^6 parasites were boiled for 5 min in 25 μ l of sample buffer (62.5 mM Tris · HCl/2% SDS/10% glycerol/5% 2-mercaptoethanol/0.003% Bromophenol blue, pH 7.2). Subsequently, the samples were loaded onto a SDS-12.5% polyacrylamide gel and electrophoresis was carried out according to standard methods [19,28]. The samples were then transferred to nitrocellulose by electroblotting as described [29]. The blot was then washed for 5 min in PBST (phosphate-buffered saline (PBS) supplemented with 0.1% Tween-20) followed by blocking of the unspecific binding sites by incubation for 1 h in PBST buffer supplemented with 3% BSA. Visualization of the respective antigens was performed as described [9].

2.7. Indirect immunofluorescence assay (IFA)

Sporozoites from salivary glands were counted and diluted to 1000 sporozoites per 10 μ l of PBS. Drops of 20 μ l were pipetted in each of the 12 wells of multitest slides, air dried and stored at -70°C . Sera were diluted in PBS and 20 μ l of the appropriate dilutions were applied to the wells containing sporozoites. After 20 min incubation in a moist chamber at 37°C , the slides were washed thrice with PBS. Fluorescein-conjugated goat anti-rabbit or rabbit anti-mouse IgG antiserum was then added at a dilution of 1:50 (in 0.05% Evans Blue) and the slides were incubated for another 20 min. After an additional wash in PBS, 50% glycerol in PBS was added to the samples and they were analyzed by fluorescence microscopy.

2.8. Suspension indirect immunofluorescence assay (SIFA)

For SIFA, 100 000 sporozoites purified by the urografin gradient method were suspended in 10 μ l of PBS and incubated at room temperature for 20 min with the appropriate antiserum. Following low-speed centrifugation the sporozoites were washed

twice with 100 μ l of PBS prior to incubation for 20 min at room temperature with 10 μ l of goat anti-rabbit or rabbit anti-mouse FITC-conjugated antiserum at a dilution of 1:50 (in 0.05% Evans Blue). After several washings with PBS, samples of 10 μ l (100 000 sporozoites) in PBS were applied to slides and covered by coverslips and they were analyzed by fluorescence microscopy.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated with 1 μ g per well of GST/B-D, a Pfs16-encoded polypeptide fused to the C terminus of *Schistosoma japonicum* glutathione S-transferase, produced in *E. coli* [9]. Assays were performed as described [9]. Titers of 1:100 or less were considered negative.

2.10. Sporozoite invasion *in vitro*

Tests of inhibition of sporozoite invasion (ISI) were carried out both with human hepatoma (HepG2-A16) cells and with primary cultures of human hepatocytes, propagated on glass coverslips (diameter 15 mm). Primary cultures of human hepatocytes were obtained as described previously [30]. HepG2-A16 cells were grown at 37°C in 5% CO_2 in air to a confluent monolayer in DMEM supplemented with 2 mM glutamine/10% fetal calf serum/100 U each of penicillin and streptomycin per ml medium. The culture medium was then replaced by DMEM without supplements and (anti)sera were diluted in the appropriate medium and added to the cell cultures. To the HepG2-A16 cells (350 000) and primary human hepatocytes (175 000) 30 000 and 150 000 sporozoites, respectively, were then added per coverslip and incubation was continued for 3 h at 37°C in 5% CO_2 . Subsequently, the coverslips were washed several times with PBS. The cell monolayers were then fixed with methanol (10 min), followed by three washes with PBS. Penetrated intracellular sporozoites surrounded by a membrane were revealed by an immunoperoxidase antibody assay using a monoclonal antibody to *P. falciparum* CS (3sp2) as described [26,31]. All experiments were carried out at least in duplicate.

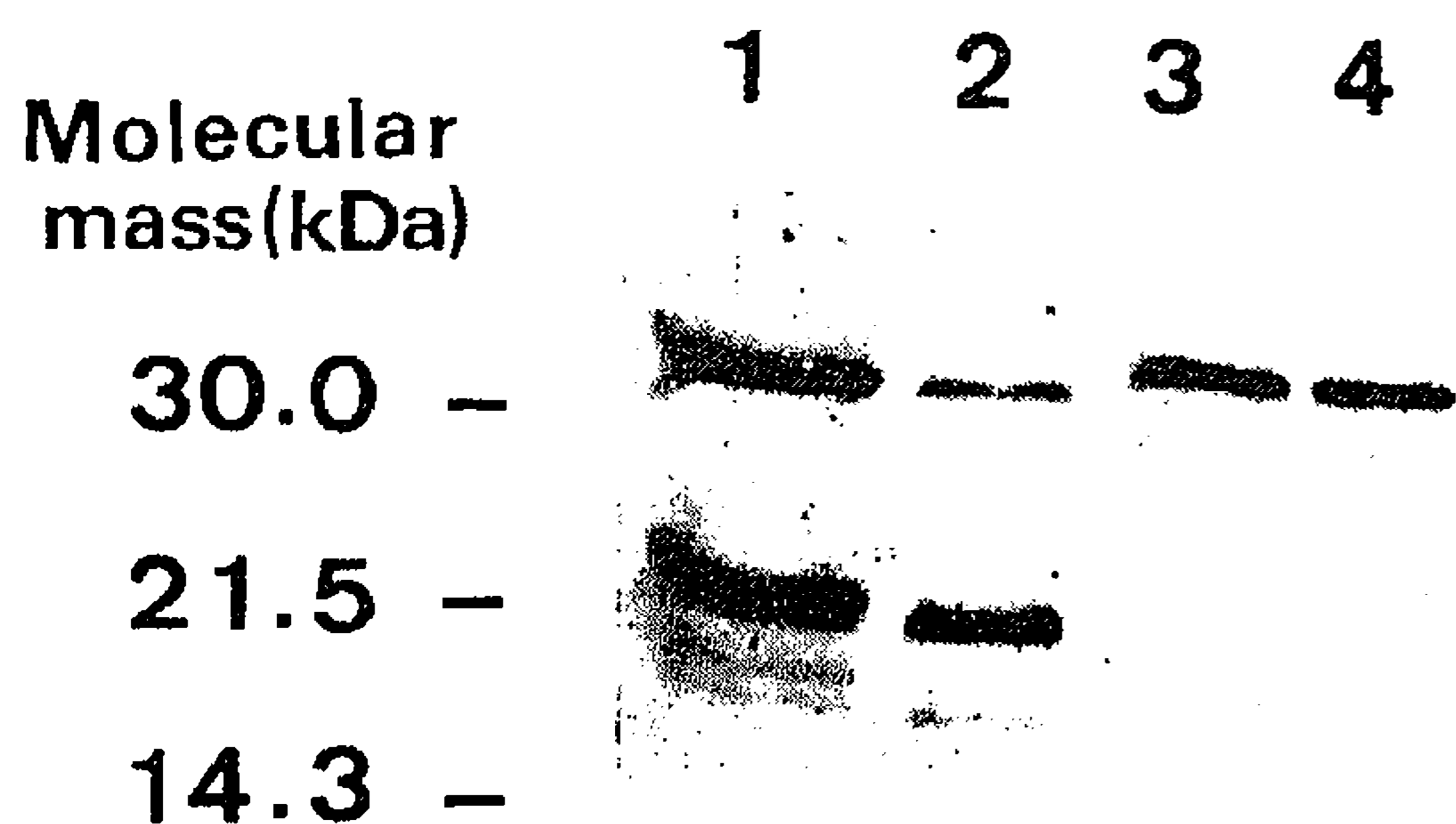


Fig. 2. Immunoblot analysis of recombinant proteins synthesized in Y1295 yeast cells transformed with expression cassette Tcd5/16-S-*Xho*I. Proteins that react with HBsAg-specific monoclonal antibody HBS1 are shown in lanes 1 and 2. Proteins that react with Pfs16-specific antiserum K37S8 are shown in lanes 3 and 4. Per lane 50 μ g of total cellular protein was applied.

2.11. Transmission-blocking assay (TB assay)

Mice and rabbit sera were tested for transmission blocking activity in a bioassay by membrane feeding of cultivated gametocytes of NF54 to female mosquitoes in the presence of test or control sera [32].

2.12. Immunoelectron microscopy

Immunoelectron microscopy was used to localize the Pfs16 antigen in the exo-erythrocytic stages of *P. falciparum*. Immunoelectron microscopy was performed as described [33].

2.13. Animals and immunization

Two Balb/c mice (M3 and M4) and New Zealand rabbits (R38 and R39) were immunized with purified recombinant yeast particles (Pfs16-S,S) composed of the recombinant proteins Pfs16-S and HBsAg. Alum was used as adjuvant. Rabbits were immunized via subcutaneous injection in weeks 0, 3 and 6 with 50 μ g of recombinant protein. Mice were immunized in weeks 0, 3 and 7 with 20 μ g of yeast particles. Serum was obtained from the animals two weeks after the third immunization (R38S3, R39S3, M3S3, M4S3). Rabbits R57 and R64 were inoculated intravenously in weeks 0 and 7 with $(1-2.5) \times 10^7$ plaque forming units of the recombinant Pfs16 vaccinia virus vSC11/16 and serum was obtained 2 weeks after the second inoculation (R57S2, R64S2). Several weeks after the third Pfs16-S,S boost rabbit

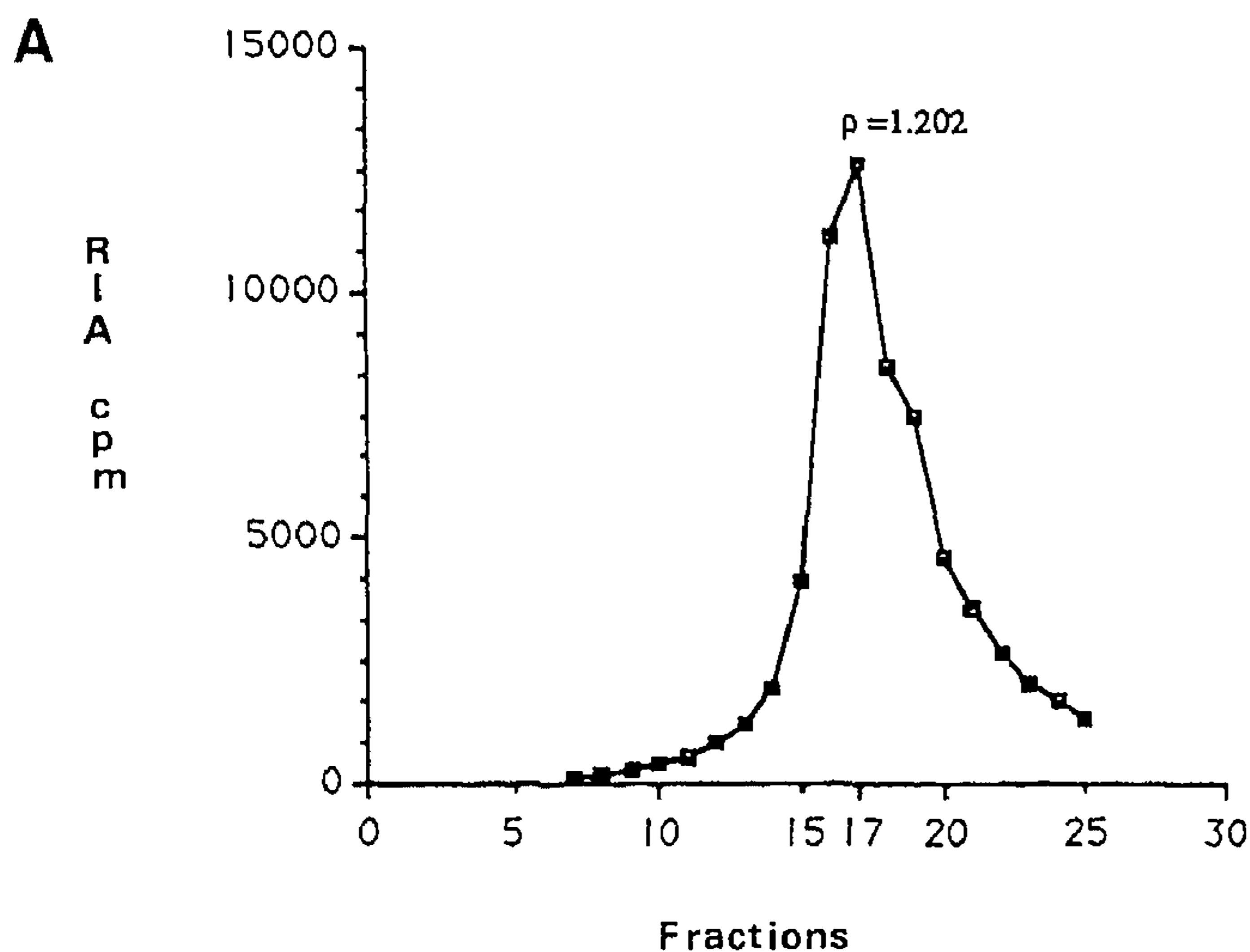


Fig. 3. CsCl gradient centrifugation of crude cell extracts prepared from yeast Y1655 cells producing recombinant Pfs16-S and HBsAg proteins. Tube fractions are numbered from bottom to top. Fractions were analyzed for their HBsAg activity by the radioimmunoassay AUSRIA II (A), and were analyzed by immunoblot analysis using K37S8 (B) and HBS1 (C) to demonstrate the production of recombinant Pfs16-S and HBsAg proteins.

R39 was also infected subcutaneously with 10^7 plaque forming units of the recombinant vaccinia virus and serum was collected 22 days after inoculation (R39v22). As controls, mouse (M2S2) and rabbit (R8S2) antisera raised against recombinant fusion proteins consisting of CS-epitopes and HBsAg produced in yeast [13] were used. Pre-sera from mice M2, M3 and M4 (M1S0) and rabbits R8, R38, R39, R57, R64 (R8S0, R38S0, R39S0, R57S0, R64S0) were taken as controls from all mice and rabbits just before the first immunizations. All antisera were

used in ELISA, Western blot analysis, IFA, SIFA, TB assay and ISI.

3. Results

3.1. Identification of recombinant proteins synthesized in yeast

To study the expression of Pfs16-S and HBsAg in yeast Y1655 cells cellular proteins were analyzed by

Table 1

Summary of reactivities of various mouse (M1S0, M2S2, M3S3 and M4S3) and rabbit antisera (R8S0, R8S2, R38S0, R38S3, R39S0, R39S3, R39v22, R57S0, R57S2, R64S0 and R64S2) in ELISA, IFA, SIFA and immunoblotting with *P. falciparum* gametocyte and sporozoite proteins

Serum	ELISA	IFA	SIFA	SWB		GWB
				Pfs16 ^a	CS ^a	
M1S0 (pre-serum)	< 1:100	–	–		ND	ND
M2S2 (CS + HBsAg)	< 1:100	+++	+++		ND	ND
M3S3 (Pfs16-S,S)	1:16000	++	+		ND	ND
M4S3 (Pfs16-S,S)	1:4000	++	+		ND	ND
R8S0 (pre-serum)	< 1:100	–	–	–	–	–
R8S2 (CS + HBsAg)	< 1:100	+++	++	–	+++	–
R38S0 (pre-serum)	< 1:100	–	–	–	–	–
R38S3 (Pfs16-S,S)	1:2000	++	+	+	–	++
R39S0 (pre-serum)	< 1:100	–	–	–	–	–
R39S3 (Pfs16-S,S)	1:1000	++	+	+	–	++
R39v22 (Pfs16-S,S + vSC11/16)	1:1 × 10 ⁶	+++	++	++	–	+++
R57S0 (pre-serum)	< 1:100	–	–	–	–	–
R57S2 (vSC11/16)	1:19200	+	+/-	++	–	+++
R64S0 (pre-serum)	< 1:100	–	–	–	–	–
R64S2 (vSC11/16)	1:76800	+	+	++	–	+++

The symbols –, +/-, +, ++ and +++ reflect an increasing intensity of reactions. SWB, sporozoite Western blot analysis; GWB, gametocyte Western blot analysis; ND, not determined.

^a Reactivity with Pfs16 or CS protein.

immunoblot analysis using both a monoclonal antibody HBS1, specific for the HBsAg monomer, and a polyclonal antiserum K37S8, raised against the recombinant Pfs16 fusion protein produced in *E. coli* [9]. As shown in Fig. 2 the monoclonal antibody HBS1 reacted with two proteins with molecular masses of 22 and 32 kDa, respectively, while the Pfs16-specific polyvalent antiserum reacted only with the 32-kDa protein. From these results it can be concluded that in Tcd5/16-S-*Xho*I transformed yeast Y1295 cells the recombinant proteins Pfs16-S and HBsAg are produced.

Studies on the expression of hepatitis B surface antigen coding sequences in yeast have demonstrated that these proteins spontaneously aggregate in virus-like particles with a diameter of 22 nm which readily can be separated from other proteins by CsCl density gradient centrifugation [25] exhibiting a density of 1.2 g cm^{-3} . These characteristics are also observed for the particles found in human patients after a hepatitis B virus infection. To study whether also the proteins Pfs16-S and HBsAg are assembled in 22 nm particles crude cell extracts of Y1655 cells were subjected to CsCl equilibration centrifugation [24]. After centrifugation fractions were collected by puncturing the bottom of the tube. Subsequently, part of each fraction was analyzed by the radioimmunoassay AUSRIA II for HBsAg antigenic activity and another part was analyzed by Western blot analysis for the presence of Pfs16 specific antigens (Fig. 3). In addition, the density of each fraction was determined. Since for recognition in the AUSRIA II assay the HBsAg antigen must have a native conformation, the data of Fig. 3A show that fractions

15–20 contain most of the native HBsAg. The average buoyant density of the particles present in these fractions was found to be 1.202. To estimate the efficiency of assembly of both HBsAg and Pfs16-S into 22 nm particles, immunoblot analysis of the gradient fractions was also performed with sera HBS1 and K37S8 that recognize linear epitope(s) on the HBsAg and Pfs16 monomers, respectively. Fig. 3B and 3C show that most of the HBsAg and Pfs16-S proteins are found in fractions 15–20, demonstrating that the results of the immunoblot analysis follow the data of the AUSRIA II radioimmunoassay (Fig. 3A). From the results obtained from the immunological analysis and from the measurements of the refraction indices it can be concluded that the proteins Pfs16-S and HBsAg assemble into 22-nm particles (Pfs16-S,S) and as efficient as native HBsAg [25].

3.2. Immunogenicity of Pfs16-S,S particles

To evaluate the immunogenicity of purified Pfs16-S,S particles, mice and rabbits were immunized as indicated in Materials and methods. Subsequently, serum samples were analyzed for the presence of Pfs16-specific antibodies by an ELISA. Titers up to 1:16 000 were obtained (Table 1). Western blot analysis showed that sera R38S3 and R39S3 specifically reacted with a 16-kDa protein present in total protein extracts of both gametocytes and sporozoites. Serum R8S2, which was raised against yeast particles containing HBsAg and CS epitopes, only reacted with the CS protein in a sporozoite Western blot. As indicated in Table 1, indirect immunofluorescence assays revealed that all Pfs16-specific anti-

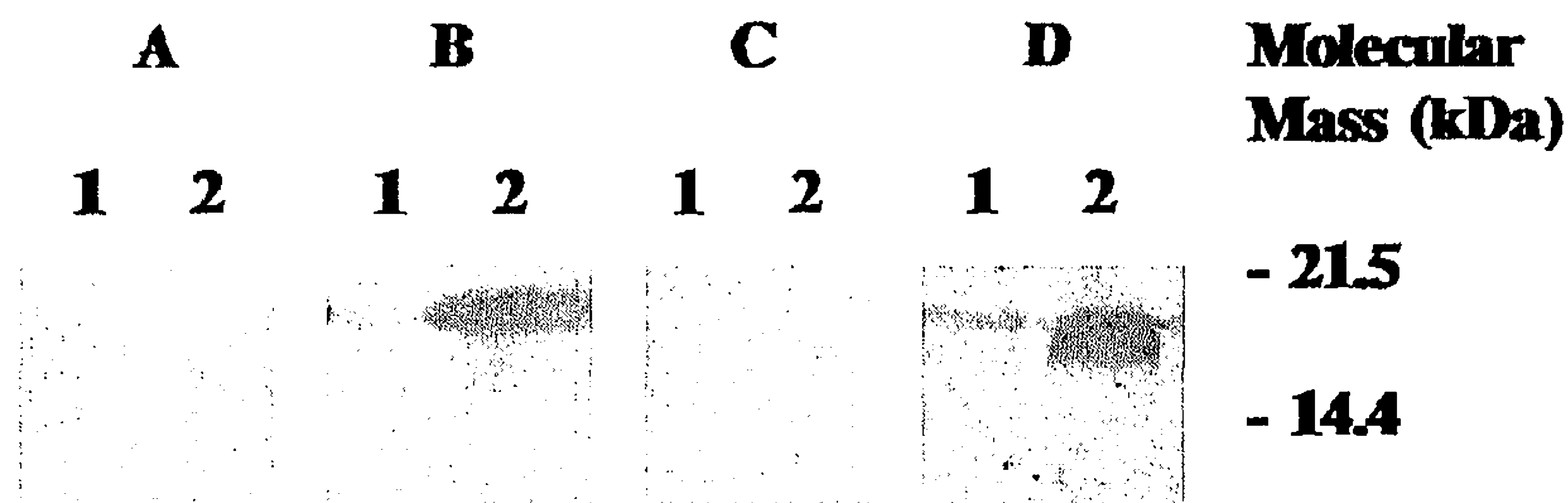


Fig. 4. Western blot analysis of protein extracts prepared from sporozoites (lane 1) and gametocytes (lane 2) using rabbit antiserum R57S2 (B), negative control serum R57S0 (A), antiserum R39v22 (D) and control pre-serum R39S0 (C). Antiserum R57S2 was collected two weeks after the second inoculation with recombinant vaccinia virus vSC11/16. Antiserum R39v22 was recovered at day 22 after infection with recombinant vaccinia virus vSC11/16 of rabbit R39 which was already immunized thrice with yeast hybrid particles Pfs16-S,S.

sera reacted with sporozoites in an IFA and fluorescence on the surface was observed when the SIFA was performed with unfixed live sporozoites, although the intensities were less than obtained after staining with the CS-specific antisera M2S2 and R8S2. As a control, pre-immune sera were used. They did not give any reaction on the Western blots and there was no fluorescence in IFA or SIFA (Table 1). From these results it can be concluded that as a result of vaccination with the Pfs16-S,S particles

Pfs16-specific antibodies are induced. Moreover, the present study confirms and extends earlier observations that the Pfs16 antigen is present on the surface of sporozoites.

3.3. Production of Pfs16 in recombinant vaccinia virus-infected cells

Expression of the heterologous gene in cells infected with recombinant vaccinia virus vSC11/16

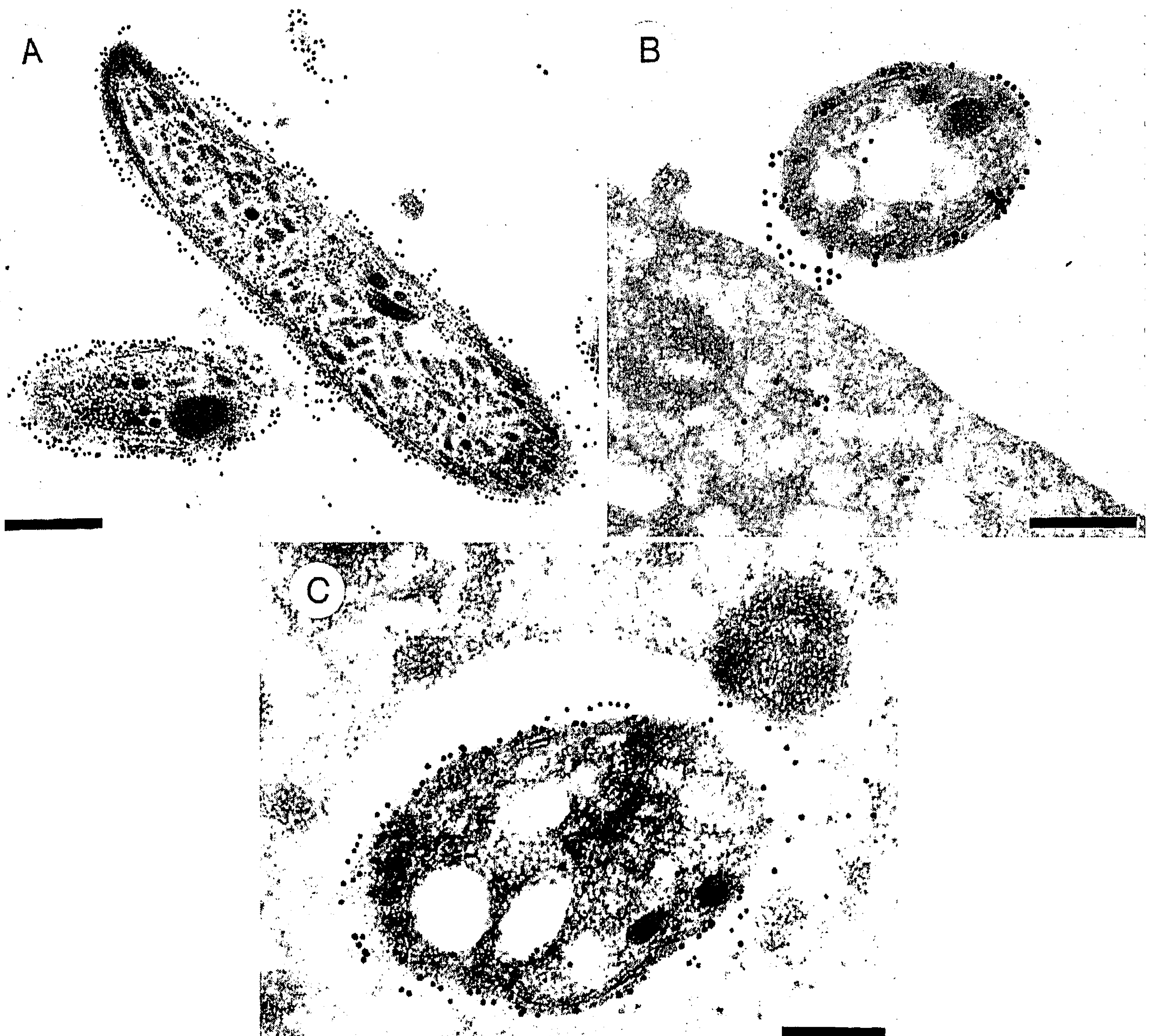


Fig. 5. Localization of the Pfs16 antigen by immunoelectron microscopy utilising antiserum R57S2. (A) Sporozoites in mosquito salivary gland, demonstrating the gold label abundantly present at the surface. (B) Sporozoite in close contact with a human hepatoma cell. (C) Liver-stage parasite in HepG2-A16 cell after infection by sporozoites. Gold label is primarily associated with the outer surface of the exoerythrocytic schizont. Bars = 0.4 μm .

Table 2

Effect of various mouse (M1S0, M2S2, M3S3, M4S3) and rabbit antisera (R8S0, R8S2, R38S0, R38S3, R39S0, R39S3, R39v22, R57S0, R57S2, R64S0, R64S2) on sporozoite invasion in HepG2-A16 cells and human hepatocytes

Serum	ISI					
	HepG2-A16			Primary hepatocytes		
	dilution	#inv.sp.	%ISI	dilution	#inv.sp.	%ISI
M1S0 (pre-serum)	1:100	72	0	1:100	64	0
	1:200	119	0			
	1:1000	151	0			
	1:2000	308	0			
	1:10000	335	0			
M2S2 (CS + HBsAg)	1:100	7	90	1:100	56	12.5
	1:200	4	97			
	1:1000	17	89			
	1:2000	64	79			
	1:10000	153	57			
M3S3 (Pfs16-S,S)	1:100	ND	ND	1:100	36	44
	1:200	ND	ND			
	1:1000	70	54			
	1:2000	210	32			
	1:10000	262	26			
M4S3 (Pfs16-S,S)	1:100	6	92	1:100	21	67
	1:200	20	83			
	1:1000	60	60			
	1:2000	330	0			
	1:10000	390	0			
R8S0 (pre-serum)	1:100	90	0		ND	
R8S2 (CS + HBsAg)	1:100	3	96		ND	
R38S0 (pre-serum)	1:100	168	0		ND	
	1:1000	197	0			
	1:2000	209	0			
	1:10000	212	0			
R38S3 (Pfs16-S,S)	1:100	55	67		ND	
	1:1000	101	49			
	1:2000	129	38			
	1:10000	132	38			
R39S0 (pre-serum)	1:100	178	0		ND	
	1:1000	162	0			
	1:2000	160	0			
	1:10000	159	0			
R39S3 (Pfs16-S,S)	1:100	31	83		ND	
	1:1000	88	46			
	1:2000	98	38			
	1:10000	149	0			
R39v22 (Pfs16-S,S + vSC11/16)	1:100	7	96		ND	
	1:1000	45	75			
	1:2000	72	61			
	1:10000	145	22			
R57S0 (pre-serum)	1:100	85	0		ND	
R57S2 (vSC11/16)	1:100	33	62		ND	
R64S0 (pre-serum)	1:100	34	0		ND	
R64S2 (vSC11/16)	1:100	54	0		ND	

More information about these sera is given in Materials and methods. ISI is expressed as percentage of invasion reduction as compared to the pre-serum. #, number of sporozoites that were able to invade HepG2-A16 cells or primary hepatocytes.

was analyzed by Western immunoblots. Western blots were prepared of cell extracts and incubated with Pfs16-specific polyclonal antiserum K37S8 [9]. In recombinant vaccinia virus infected cells but not in uninfected cells or in cells infected with wild type virus, a protein with a molecular mass of approx. 16 kDa could be demonstrated (data not shown).

3.4. Production of Pfs16-specific antisera with recombinant vaccinia virus

To determine whether the vaccinia virus recombinants were able to induce specific and strong immune responses to the Pfs16 protein, rabbits were infected twice with recombinant vaccinia virus vSC11/16. After collection the antisera (R57S2 and R64S2) were first analyzed for the presence of Pfs16-specific antibodies by ELISA. As shown in Table 1, high antibody titers were reached. Subsequently, the antisera were analyzed for the presence of antibodies reactive to Pfs16 by Western blot analysis of whole lysates prepared from gametocytes and sporozoites (Fig. 4). As shown in Fig. 4, Western blot analysis demonstrated that the antisera R57S2 and R64S2 reacted specifically with the 16-kDa protein as present in sporozoites and gametocytes. Furthermore, IFA and SIFA revealed that the antisera also reacted specifically with antigens present on fixed and live *P. falciparum* sporozoites (Table 1), respectively. Moreover, immunoelectron microscopy demonstrated the presence of the Pfs16 antigen at the surface of sporozoites, as well as at the outer surface of exoerythrocytic schizonts collected after invasion of HepG2-A16 cells by sporozoites (Fig. 5). No reaction was observed with the pre-sera R57S0 and R64S0.

The results of an additional infection of rabbit R39 with recombinant vaccinia virus vSC11/16 several weeks after the third Pfs16-S,S boost are also presented in Table 2. The ELISA results showed that a very high Pfs16-specific antibody titer was obtained when compared to the titers seen after immunization with Pfs16-S,S or vSC11/16 alone. Antiserum R39v22 also reacted with the Pfs16 antigen on both a gametocyte and a sporozoite Western blot (Fig. 4). We could also demonstrate a strong reactiv-

ity of the serum with the surface of sporozoites in the IFA and SIFA.

3.5. Transmission-blocking activity

All mice and rabbit sera were tested for transmission-blocking activity in a standard bioassay as described in Materials and methods. None of the antisera showed transmission-blocking activity.

3.6. Inhibition of sporozoite invasion (ISI) in vitro

We used the inhibition of sporozoite invasion (ISI) into cultured cells assay [31,34] to investigate whether sera from mice and rabbits vaccinated with yeast Pfs16-S,S particles and/or recombinant vaccinia virus were able to neutralize sporozoite infectivity. Positive controls were antisera raised in mouse (M2S2) and rabbit (R8S2) against yeast particles containing CS and HBsAg epitopes. Antisera from mice (M3S3 and M4S3) and rabbits (R38S3 and R39S3) immunized with yeast Pfs16-S,S particles significantly reduced invasion of sporozoites into HepG2-A16 cells, although their capacity to reduce invasion was less than sera to CS protein (Table 2). However, when primary hepatocytes were used, sera M3S3 and M4S3 were significantly more active than serum M2S2 against CS protein. When sera from rabbits immunized with recombinant vaccinia virus vSC11/16 were tested by ISI, only R57S2 had ISI activity, whereas R64S2 was inactive. However, the rabbit pre-serum R64S0 itself significantly lowered the numbers of invaded sporozoites (Table 2), which may have obscured R64S2 activity. When rabbit R39 previously immunized with recombinant yeast Pfs16-S,S particles was boosted with recombinant vaccinia virus vSC11/16, the ISI activity of this antiserum (R39v22) increased and was similar to rabbit serum R8S2 against CS protein. This correlates with the efficient boosting capacity of the recombinant vaccinia virus shown by anti-Pfs16 ELISA activities (Table 1). None of the antisera showed transmission-blocking activity.

4. Discussion

A sporozoite vaccine is expected to block either sporozoite invasion of hepatocytes or its subsequent intrahepatic development. Identification of sporo-

zoite ligands might permit development of vaccines that elicit antibodies that block ligand recognition of hepatocyte receptors. It is likely that a series of molecular interactions are critical for sporozoite invasion. CS protein Region I and Region II, found in both CS protein and TRAP, have been shown to specifically bind to hepatoma cells in vitro, and antibodies to these regions block sporozoite invasion [35–37]. Recently, van Pelt et al. [26] have suggested that *P. falciparum* sporozoite invasion of human hepatocytes involves two distinct 20 and 55-kDa membrane proteins, although the sporozoite ligands have not been identified. The results reported here suggest that the highly conserved Pfs16 protein found in both sexual stages and sporozoites [9,10] also might be involved in sporozoite invasion.

To obtain more information on the precise biological function of the 16-kDa protein and to explore its role as a vaccine candidate, immunization studies were performed using recombinant Pfs16 vaccinia virus, and a Pfs16 fragment fused to the HBsAg, and expressed as virus-like particles in the yeast *Saccharomyces cerevisiae*. In line with previous findings [9] all antisera reacted with a 16-kDa protein in Western blots prepared from gametocyte or sporozoite extracts. In the suspension immunofluorescence assay, antibodies to the recombinant Pfs16 product reacted with the surface of the sporozoite, suggesting that these sera recognized the authentic native Pfs16 antigen. Furthermore, antibodies to the protein constructs specifically reduced in vitro invasion of human hepatoma HepG2-A16 cells and primary human hepatocytes. Of interest was our finding that mouse anti-Pfs16 antibodies were more active in blocking invasion of human hepatocytes than anti-CS protein antibodies. We have previously demonstrated that sera from human volunteers immunized with CS protein vaccines and protected to sporozoite challenge blocked sporozoite invasion of hepatoma HepG2-A16 cells but not human hepatocytes [34]. Thus, Pfs16 may represent a candidate malaria vaccine designed to elicit sporozoite neutralizing antibodies.

Immunoelectron microscopy experiments using antisera R57S2 and R64S2 from rabbits infected with recombinant vaccinia virus confirm that Pfs16 is localized on the surface of sporozoites (Fig. 5A) and appears to accumulate between hepatoma cell

and the attached sporozoite (Fig. 5B). We also found that the Pfs16 is localized on newly invaded sporozoites as well as *P. falciparum* liver stage parasites. The liver stages are attractive targets [38–40] for vaccine development because they are susceptible for immune attack for much longer than sporozoites, i.e., at least five days compared to a few minutes [40,41]. Moreover, both CD4+ and CD8+ T cells have been shown to attack malaria-infected hepatocytes, either by direct cytotoxicity or indirectly by cytokines, suggesting that *P. falciparum* epitopes are expressed on the surface of infected hepatocytes in association with MHC Class-I or -II molecules [40,42–44]. The presence of Pfs16 during *P. falciparum* liver-stage development suggests that Pfs16 vaccines could also elicit cytotoxic T-cell responses directed to *P. falciparum*-infected hepatocytes.

None of the antisera showed transmission-blocking activity, indicating that Pfs16 probably does not play a critical role in the process of transmission of the parasite from the human host to the mosquito vector. Comparable results were observed in previous Pfs16 immunization experiments [9] and this is also in agreement with Baker et al. [11] and Bruce et al. [12] who demonstrated that Pfs16 is located on the parasitophorous vacuole membrane of the gametocyte and not on the plasma membrane of gametes. Since antigens on the gamete plasma membrane are the most likely targets of transmission-blocking antibodies, the absence or low expression of the Pfs16 antigen on the gamete plasma membrane may explain lack of transmission-blocking activity. However, the location of Pfs16 on the parasitophorous vacuole membrane suggests that this antigen may play a role during gametocytogenesis or is involved in the emergence of macrogametes from the parasitophorous vacuole membrane. The possible role of Pfs16 in these processes is currently under investigation.

In conclusion, the present study confirms that the Pfs16 antigen is expressed on *P. falciparum* sporozoites. Pfs16 may be involved in sporozoite invasion of hepatocytes, since Pfs16-specific antibodies reduced sporozoite invasion into human hepatoma cells and human hepatocytes. Since Pfs16 is also expressed during *P. falciparum* liver-stage development, Pfs16 may represent a candidate antigen for malaria vaccine development.

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