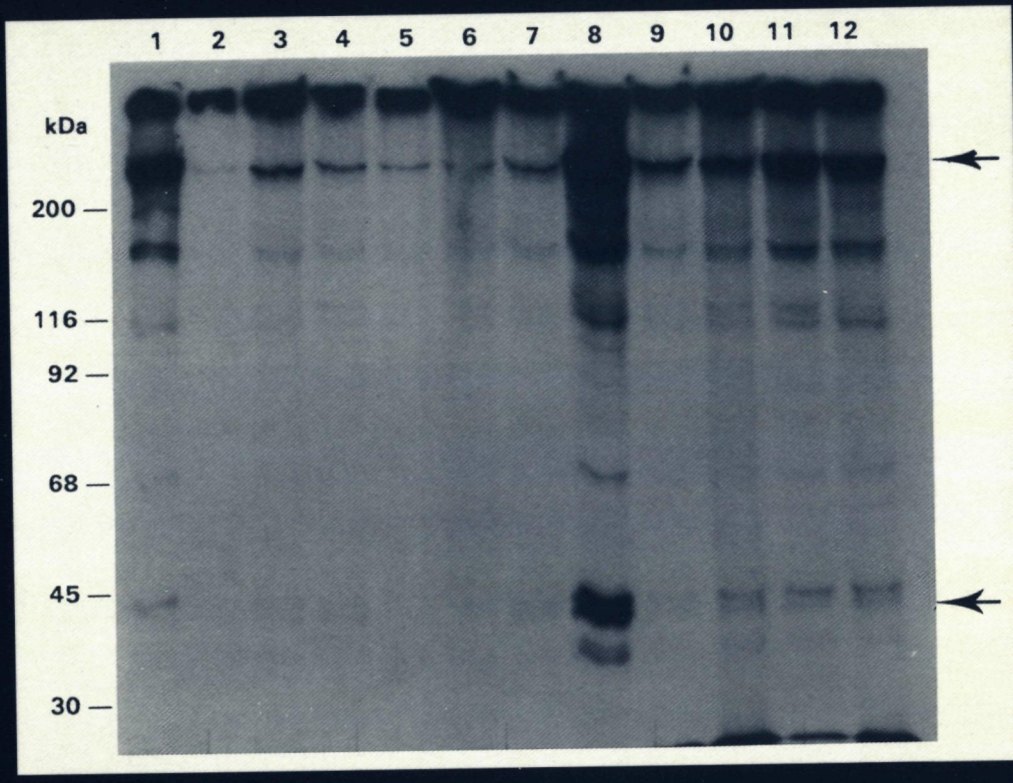
Plasmodium falciparum:

Relevance of human antibodies for blocking transmission of the parasite from man to mosquito



Will F. G. Roeffen

Plasmodium falciparum:

Relevance of human antibodies for blocking transmission of the parasite from man to mosquito.

Will F. G. Roeffen

Cover illustration: Autoradiograph of immunoprecipitations from NP-40 extract of protein surface radio-iodination of macrogametes/zygotes with sera of gametocyte carriers and analyzed by SDS-page under non-reducing conditions. Lane 1 mAb 18F25, lane 2-11 randomly selected gametocyte carriers and lane 12 the positive control (St1).

CIP-data, Koninklijke Bibliotheek, Den Haag

ISBN 90 373 0348 X

1996
Drukkerij: Ponsen & Looyen, Wageningen

Plasmodium falciparum: Relevance of human antibodies for blocking transmission of the parasite from man to mosquito.

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op dinsdag 12 november 1996
des namiddags om 1.30 uur precies

door

Wilhelmus Franciscus Gerardus Roeffen

geboren op 11 oktober 1950 te Maasniel

Promotor: Prof. Dr. J.H.E.Th. Meuwissen

Co-promotores: Dr. R.W. Sauerwein
Dr. W. M.C. Eling

The studies presented in this thesis were performed at the Department of Medical Microbiology, section Parasitology, University Hospital St. Radboud, Nijmegen, The Netherlands

These studies were in part supported by grants NI/89/078 and NL002701) from the Dutch Ministry for Development Cooperation (DGIS/SO) and by grants TS2-M-0274 and TS3-C193-0229 from the European Economic Community

Science is always wrong: it never solves a problem without creating ten more.
(GB Shaw, 1856-1950)

aan Ine en Katja
aan mijn ouders

CONTENTS page

CHAPTER 1

GENERAL INTRODUCTION

1.1 General aspects of a malaria infection

1.2 Biology of the sexual stage of *Plasmodium falciparum*

1.3 Immunity to the sexual stages

1.4 Target antigens of transmission-blocking antibodies

1.5 Outline of this thesis

1.1 General aspects of a malaria infection

Malaria is a predominant public health problem in tropical areas with rainfall throughout the year. Human disease is caused by parasites in red blood cells that belong to four species of *Plasmodium*: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*. Malaria tropica caused by *P.falciparum* is a main cause of severe illness and death. In recent years the problem has been aggravated by the develop-ment of resistance against the drugs that were in common use for the treatment of this infectious disease. Worldwide it is estimated that there are about 300-500 million clinical cases of malaria of which about 90% occur in Africa alone and that there are between 1.5 - 3 million deaths per year with 800.000 deaths in African children [64].

The malaria parasites are transmitted from man to man by several species of *Anopheline* mosquitoes. The wide distribution of malaria is attributable to the adaptability and breeding potential of the *Anopheles* mosquito. The greater part of the population is infected with the parasite in regions where the conditions favour transmission throughout the year; this situation is indicated as hyperendemic malaria. Under those circumstances the indigenous people are continuously exposed to infectious mosquito bites. The clinically severe malaria infections occur in people which are insufficiently immune against the parasites. It is evident that protective immunity can be developed through natural exposure to the malaria parasites. In areas of intense transmission, the bulk of morbidity and mortality as a result of malaria infection occurs in infants, children under five years of age and pregnant women. The children still have to establish their own active species and strain specific antimalarial immunity and women experience during pregnancy a physiologic period of immune depression. The severity and frequency of malaria attacks gradually decline with age until only relatively mild and sporadic episodes occur from adolescence onwards. Even then, the acquired immunity is less than solid, and occasional episodes of parasitaemia and disease persist throughout life.

It is a common view that malaria immunity is build on the individual experience with various types of locally available malaria parasites species and their strains[&] [18]. Those who survive the first years of repeated infections develop clinical immunity and premunition [53]. According to this concept a status of a persisting low grade parasitic infection of the blood provides a gradually acquired immune protection against superinfection with the locally present parasite species and strains. Radical treatment with antimalarial drugs

---

[&] The concept "parasite strain is still ill-defined and describes within a species a geographically restricted subpopulation of parasites which share one or more characteristics of interest, e g closely related antigenicity.

results in the loss of parasites and if premune people are no longer exposed to reinfection effective premunition is lost within a period of several months In persons who have acquired through regular infections parasitemia may exist without malaria illness In premune people exposed to regular infections the chronic parasitemia is not accompanied by major acute clinical symptoms. These people contribute to maintaining the infectious malaria reservoir in the local mosquito population in this way Therefore migrants from geographically different regions where other malaria parasite strains are present as well as non-immune visitors remain at risk for acquisition of clinical infections.

1.2 Biology of the sexual stage of *Plasmodium falciparum*

The complex life cycle of the malaria parasite, comprising several stages which are morphologically, biochemically and antigenically distinct is schematically drawn in Figure 1 Natural malaria infection develops after injection of sporozoites (**A**) into the host's bloodstream when a female *Anopheles* mosquito takes a bloodmeal. The sporozoites rapidly leave the circulation and invade host liver cells and become exoerythocitic schizonts (**B**) leading to the development of thousands of merozoites On release from the disintegrating liver cells into the bloodstream, the merozoites (**C**) invade erythrocytes, mature through an erythrocytic trophozoite (**D**) and finally erythrocytic schizonts divide into 8 to 32 new merozoites (**E**) which are released as daughter cells when the erythrocyte bursts These merozoites invade other erythrocytes, thereby continuing the erythrocytic cycle of parasite multiplication, which is the only stage of the infection that is accompanied by clinical manifestations of the disease and which takes 48 hours for completion (72 hours in *P malariae*) During the process of erythrocytic schizogony some parasitized erythrocytes are induced to differentiate from asexual into sexual stages of the parasite (**F**), the so called male and female gametocytes [28,6]. The mechanism of this induction is as yet unknown [47,12]. The immature gametocytes of *P falciparum* mature while adherent to the endothelial cells of small vessels The development to morphological and functional maturity [47,57,48,12] requires at least 7 days and they become microscopically detectable in bloodsmears some 10 days after the asexual parasitemia became patent Gametocytes are apathogenic and become functionally active only when they are ingested by an *Anopheles* mosquito after taking an infected bloodmeal The mature and functional gametocytes in the mosquito midgut are stimulated to transform into extracellular male and female gametes (**G**) under influence of changes in temperature, pH and CO_2 [58,56,8-,12] The microgametocyte (male form) forms eight highly motile microgametes [29] and the macrogametocyte increases the cell volume and 'rounds up' [55,12] Fertilization of the macrogametes ensues, and is complete within 10 - 30 minutes after ingestion of a

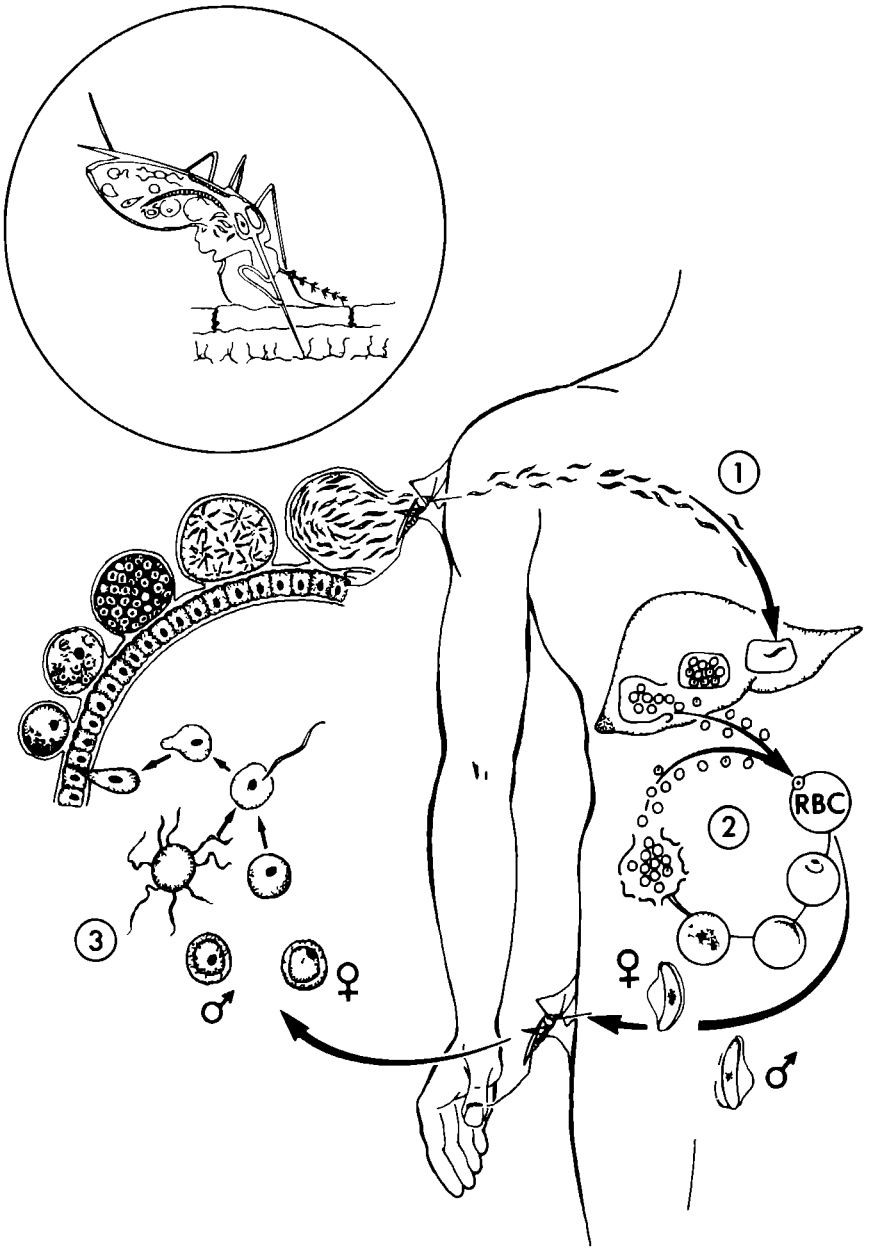


Figure 1 Life cycle of the human malaria parasite *Plasmodium falciparum*

blood meal [58,55,12]. The fertilised zygotes transform into retort cells and further into motile ookinetes (**I**) that makes contact to the cells lining the stomach wall.
The ookinetes penetrate through or between the epithelial cells of the stomach wall and finally rest on inner side of the lamina basalis of the epithelial cells on the outside surface of the stomach wall where they develop into oocysts (**J**) [36,37]. Within the oocyst thousands of sporozoites are formed (**K**) which, upon rupture of the oocyst, migrate via the haemocoel to the salivary glands.

1.3 Immunity to the sexual stages

Many patients show gametocytes for the first time about three weeks after a sporozoite induced infection, or two to four days after the first day of a recrudescence. Shute and Maryon [54] and Wilson [66] have shown that in children ≤ 5 years of a hyperendemic region, gametocytes are more abundant and the prevalence of carriers higher than in any other age group. In countries with one transmission season only, gametocytes are more abundant and seen relatively more frequently in adolescents and adults in comparison with hyperendemic regions. Thus gametocytaemias are more prevalent before clinical immunity is acquired. In addition, non-immune adults who have entered the area and become infected can become good gametocyte producers and good transmitters.

Antibodies to gametocytes of *P.falciparum* were first reported by Voller and Bray [63] using immunofluorescence. Antibodies directed against surface antigens of sexual stage parasites can be induced in the vertebrate host by immunization with gametocytes, gametes or purified antigens [7,25,9,40,10,27,4]. These antibodies propably do not have access to gametocytes in the bloodstream, where they are protected by the red blood cell membrane, but they can interact with the parasite cell surface of gametes. Four different groups of monoclonal antibodies (mAbs) were shown to be effective in reducing transmission: (1) antibodies against gamete surface antigens which propably block parasite fertilization [7,25,9,39,45]; (2) anti-gamete antibodies inducing complement-dependent lysis of gametes and fertilized zygotes [51,45,38]; (3) antibodies against ookinete surface antigens which inhibit post-fertilization growth and development [60,38,67] and (4) opsonizing antibodies showing white blood cell dependent transmission-reduction [35]. Antibodies are considered the most important immune mediators that reduce transmission but evidence has been presented that other mechanisms including phagocytic leucocytes [56], cellular immunity [26], cytokines and reactive nitrogen intermediates [44,46] may affect transmission. Transmission-blocking (TB) immunity, that is directed against the sexual stages of parasitic development, prevents the fertilization process or the further development of oocysts and no sporozoites are formed, thereby preventing spreading of the parasite [9,

27]. TB-immunity is observed in the analysis of serum of people living permanently or periodically in endemic areas and who where infected with the parasite [45,38,23]

1.4 Target antigens of Transmission-Blocking (TB) antibodies

As targets for antibodies, a number of *P falciparum* gametocyte and gamete antigens have been identified by immunoprecipitation, metabolic labelling and 'pulse and chase' experiments [42,60,62] Antigens present in blood stage gametocytes and retained in mosquito midgut on the surface of freshly emerged gametes include Pfs230, Pfs48/45 and Pfs40/10 (Table I). These are pre-fertilisation antigens and it is likely that natural immunity against such antigens is boosted by a malaria infection. During the transformation of a zygote into an ookinete a change in the composition of the parasite surface proteins occurs, i e Pfs230 and Pfs48/45 are shed from the surface and a newly synthesized 25-kDa (Pfs25) protein appears on developing zygotes, retort cells and ookinetes Up to now these proteins have been preferentially studied though other proteins were also found on the surface, e g Pfs40/10 and Pfs28

TABLE I Sexual stage proteins of *P falciparum* of which genes are expressed

Protein	Location of protein	Induction of TB	References
Pfg377	gametocyte dense bodies	no?	[3]
Pfs230	gametocyte and gamete surface	yes	[51,60,49,1-
Pfs48/45	gametocyte and gamete surface	yes	[51,60 16,1]
Pfs40/10	gamete surface	no?	[1,50]
Pfs28	gamete and ookinete surface	yes	[19]
Pfg27/25	gametocyte internal	yes?	[68,15,2]
Pfs25	gamete and ookinete surface	yes	[60,1,31]
Pfg16	gametocyte pasitophore vacuole mem-	no	[5,43]
Pf 11 1	cytoplasm of gametocyte infected RBC	yes?	[20,52]
Chitinase		yes	[17]

Pfs25 and the newly identified parasite-specific chitinase are post-fertilisation antigens expressed exclusively in the mosquito midgut The biological function of the described proteins is still not known It is assumed that antibodies against Pfs230 and Pfs48/45

interfere in the process of fertilization, since antibodies directed against these antigens block further development of the parasite [51,60]. It has been suggested that antibodies against Pfs25 interacts with a ligand of the ookinete for a receptor on the mosquito midgut wall, preventing penetration of the ookinete [60].

Epitopes of the Pfs230 surface protein.

In *P falciparum*, mAbs directed against the 230-kDa protein block the further development of the gamete or zygote only in the presence of complement [49]. Only in *P.gallinaceum* a reduced gametocyte infectivity was shown by a synergistic effect of two distinct mAbs directed against the Pfs230 analogous in the absence of complement [32]. Two epitopes on Pfs230 were identified as targets of TB-mAbs. One was shown to be universally conserved within 45 isolates; the other was demonstrable again in all isolates, though 3 of the isolates had small populations of gametocytes that did not react [21]

Epitopes of the Pfs48/45 surface protein.

A number of anti-Pfs48/45 mAbs are able to block transmission of the parasites to the mosquito vector [51,60,16,59] The immunological reactivity of the epitopes of Pfs48/45 was investigated in more detail in a two-site radio-immuno assay. Based on competition, 5 distinct antibody epitopes have been identified, however none of them being particularly associated with the capacity to induce TB [11,61,13,16,59]. These epitopes are monovalently expressed on the proteins Epitope II is subdivided into epitopes a, b and c of which a and c represent the genetic variants [16] In addition, Foo *et al.* [21] found in a serie of 33 *P falciparum* isolates from Malaysia that variation exists in epitope IIa and IIc, while epitope I, IIb, III and IV are markedly conserved. Using the PCR technique nucleotide and amino acid sequence differences were observed between the Pfs48/45 genes of 8 different strains of *P falciparum* by Kocken *et al.* [33]. The amino acid residue at position 254 in Pfs48/45 determines whether epitope IIa or IIc is expressed.

Structure of Pfs25

Pfs25 is a cysteic-rich (>10% of the residues) glyco-protein on the surface of gametes, zygotes and ookinetes Two distinct antibody binding sites have been identified, one of which is target for TB-immunity Both epitopes are conformational and dependent on the tertiary structure of the protein and on the presence of linked fatty acids, but independent of carbohydrate groups [60,22] Foo et al. [21] showed that epitope I, which is target of TB-mAbs showed some variability amongst 45 Malaysian isolates, while epitope II was conserved throughout The gene encoding Pfs25 has been isolated [31] and is characterized by the presence of four tandem epidermal growth factor (EGF)-like domains Only minimal variations in antigenic composition were found among different isolates of *P.falciparum* [30] and this would be consistent with the view that Pfs25 is not subjected to

immune pressure in the human host and does not show polymorphism

Other membrane proteins

The role in TB-immunity of several other proteins (e g Pfg377, Pfs40/10, Pfg27/25, Pfs16, Pf 11 1 and chitinase) of the sexual stages of *P falciparum* is not clear (see Table 1) and only few data about these proteins are available The mechanisms of action of the TB-antibodies which react with intracellular proteins of sexual stages such as Pfg27/25 and Pf 11 1 are not understood It has been found that a mAb against Pfg27/25 cross reacts with an epitope shared between Pfs230 and Pfs48/45 [68] and this may be the TB target of this mAb

1.5 Outline of this thesis

The gametocytes are non pathogenic in the human host and their only goal is to infect mosquitoes, thus transmitting the parasites Though *P falciparum* 230 kDa and 48/45 kDa proteins are expressed predominantly on the gamete surface, their synthesis begins at an early stage in gametocyte development This explaines the presence of antibodies to these target antigens in sera from infected individuals [41,24,14] Sexual stages can induce immune responses that may affect the outcome of the infection in the mosquito, e g , antibodies against the sexual stage specific Pfs230 and Pfs48/45 MAbs against these proteins do have TB-activity

The direct laboratory test for the analysis of *P falciparum* TB-immunity is the *in vitro* feeder assay, indicating as the bioassay based on development of oocysts in mosquitoes after membrane feeding of gametocytes in the presence of the serum control and test samples [60] It is a labourious, time-consuming and costly assay whereby only a restric ted number of sera can be tested Therefore an alternative testmethodology is needed for the assessment of TB-immunity in the field and of future TB vaccins with regard to their impact on the infectious gametocyte reservoir in the local population The objectives of the studies described in this thesis were as follows

1) the development of serological tests to study the presence of antibodies against Pfs230 and/or Pfs48/45 in sera from naturally infected individuals

2) to study the association between the presence of these antibodies and the capacity of sera to block *P falciparum* transmission.

MAbs, recognizing Pfs230 were used in specific competition ELISAs to study the associa-tion between the presence of those antibodies and TB-activity of human serum samples (chapter II) To determine whether anti-Pfs230 mAbs mediated transmission-blockade is isotype and epitope dependent, a number of isotype switch variants were prepared and selected for differences in their capacity to fix complement (chapter III) Anti-Pfs230

mAbs and anti-Pfs48/45 mAbs were used in competition ELISAs for the detection of natural antibodies in sera from gametocyte carriers and to compare these results with TB-activity of these sera in the bio-assay (chapter IV). In chapter V and VI the association was studied between TB-activity and competition-ELISA titers against different epitopes of Pfs48/45 among different isolates of *P.falciparum*. Antibody activity in competition-ELISAs and the activity of the serum in the TB-bioassay was longitudinally studied in blood samples collected from adult patients with a *P.falciparum* malaria on admission and at several time points after malaria treatment (chapter VII). A summary and general discussion is given in chapter VIII.

REFERENCES

1 ALANO P. (1991). *Plasmodium* sexual stage antigens. *Parasitology today* 7:199-203.

2 ALANO P, PREMAWANSA S, BRUCE MC & CARTER R. (1991). A stage specific gene expressed at the onset of gametocytogenesis in *Plasmodium falciparum*. *Mol Biochem Parasitol* **46**:81-88

3 ALANO P, READ D, BRUCE M, AIKAWA M, KAIDO T, TEGOSHI T, BHATTI S, SMITH DK, LUO C, HANSRA S, CARTER R & ELLIOTT JF. (1995) COS cell expression cloning of Pfg377, a *Plasmodium falciparum* gametocyte antigen associated with osmiophilic bodies. *Mol Biochem Parasitol* **74**:143-156

4 BEETSMA A., SAUERWEIN R. & ELING W. Transmission-blockade in mice infected with *P.berghei*. submitted.

5 BRUCE MC, CARTER RN, NAKAMURA K, AIKAWA M & CARTER R. (1994). Cellular localization and temporal expression of the *Plasmodium falciparum* sexual stage antigen Pfs16. *Mol Biochem Parasitol* **65**:11-22.

6 BRUCE MC, ALANO P, DUTHIE S & CARTER R. (1990). Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development *Parasitology* **100**:191-200

7 CARTER R. & CHEN DH. (1976). Malaria transmission blocked by immunization with gametes of the malaria parasite. *Nature* **293:**57-60.

8 CARTER R & NIJHOUT MM. (1977). Control of gamete formation (exflagellation) in malaria parasites. *Science* **195:**407-409

9 CARTER R, GWADZ RW & MCAULIFFE FM. (1979) *P. gallinaceum* Transmission-blocking immunity in chickens I. Comparative immunogenicity of gametocyte- and gamete-containing preparations. *Exp Paras* **47**:185-193

10 CARTER R & GWADZ RW (1980). Infectiousness and gamete immunization. Malara vol 3, ed JP Kreier, Chapter 7, pp 262-297.

11 CARTER R, BUSHELL G, SAUL A, GRAVES PM & KIDSON C. (1985). Two apparently nonrepeated epitopes on gametes of *Plasmodium falciparum* are target of transmission blocking antibodies. *Inf Immun* **50:**102-106.

12 CARTER R & GRAVES PM. (1988). Gametocytes in *Malaria. Principles and practice of malariology*. Ed. by W.H Wernsdorfer & Sir I. McGregor Vol. **1**, 253-305.

13 CARTER R, KUMAR N, QUAKYI I, GOOD M, MENDIS K, GRAVES P & MILLER L. (1988). Immunity to sexual stages of malaria parasites *Progress of allergy* **41:**193-214.

14 CARTER R, GRAVES PM, QUAKYI IA & GOOD MF. (1989). Restricted or absent immune responses in human populations to *Plasmodium falciparum* gamete antigens that are targets of malaria transmission-blocking antibodies *J Exp Med* **169:**135-147.

15 CARTER R, GRAVES PM, CREASEY A, BYRNE K, READ D, ALANO P & FENTON B. (1989). *Plasmodium falciparum*: An abundant stage-specific protein expressed during early gametocyte development. *Exp Parasitol.* **69**:140-149

16 CARTER R, GRAVES PM, KEISTER DB & QUAKYI IA (1990) Properties of epitopes of Pfs48/45, a target of transmission-blocking monoclonal antibodies, on gametes of different isolates of *P. falciparum. Parasite Immunol* **12**, 587-603.

17 CARTER R. (1992). Malaria transmission-blocking vaccins against sexual and mosquito mid-gut stages of the parasites: The current state of Development. Symposium on recombinant and synthetic vaccines. New Dehli

18 CREASY A, FENTON B, WALKER A, THAITHONG S, OLIVEIRA S, MUTAMBU S & WALLIKER D (1990). Genetic diversity of *Plasmodium falciparum* shows geographical variation. *Am J Trop Med Hyg* 42:403-413

19 DUFFY PE & KASLOW DC (1995) The addition of anti-Pfs28 antibodies to anti-Pfs25 antibodies enhances malaria transmission-blocking activity. Proceedings of the NIMR Conference, Arusha:28

20 FENG Z, HOFFMANN RN, NUSSENZWEIG RS, TSUJI M, FUJIKOKA H, AIKAWA M, LENSEN THW, PONNUDURAI R & POLONGE LG (1993). Pfs2400 can mediate antibody-dependent malaria transmission inhibition and may be the *Plasmodium falciparum* 11 1 gene product. *J Exp Med* **177**: 273-281

21 FOO A, CARTER R, LAMBROS C, GRAVES P, QUAKYI I, TARGETT G, PONNUDURAI T & LEWIS G. (1991). Conserved and variant epitopes of target antigens of transmission-blocking antibodies among isolates of *P. falciparum* from Malasia. *Am J Trop Med Hyg* **44**, 623-631

22 FRIES HCW, LAMERS MBAC, SMITS MA, PONNUDURAI T & MEUWISSEN JHETH. (1989). Characterization of epitopes on the 25 kD protein of macrogametes/zygotes of *Plasmodium falciparum. Parasite Immunol* **11**:31-45

23 GRAVES PM, CARTER R, BURKOT TR, QUAKYI IA & KUMAR N. (1988) Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera. *Parasite Immunol* **10**.209-218.

24 GRAVES PM, WIRTZ RA, CARTER R, BURKOT TR, LOOKER M & TARGET GAT. (1988). Naturally occurring antibodies to an epitope on *Plasmodium falciparum* gametes detected by monoclonal antibody based competitive enzyme-linked immunosorbent assay. *Infect Immun* **56**:2818-2821.

25 GWADZ RW. (1976). Malaria. succesful immunization against the sexual stages of *Plasmodium gallinaceum Science* **193**:1150-1151.

26 HARTE PG, ROGERS NC & TARGETT GAT (1985). Role of T cells in preventing transmission of rodent malaria *Immunology* **56**:1-7

27 HARTE PG, ROGERS N & TARGETT GAT (1985). Vaccination with purified microgamete antigens prevents transmission of rodent malaria. *Nature* **316**:258-259

28 INSELBURG J. (1983). Gametocyte formation by the progeny of single *Plasmodium falciparum* schizonts *J Parasitol* **63**:584-591.

29 JANSE CJ, PONNUDURAI T, LENSEN AHW, MEUWISSEN JHETH, VAN DER PLOEG M & OVERDULVE JP (1987) DNA synthesis in gametocytes of *Plasmodium falciparum. Parasitology* **96** 1-7

30 KASLOW DC, QUAKYI IA & KEISTER DB (1989) Minimal variation in a vaccine candidate from the sexual stage of *Plasmodium falciparum* *Mol Biochem Parasitol* **32** 101-104

31 KASLOW DC, QUAKYI IA, SYIN C, RAUM MG, KEISTER DB, COLIGAN JE, MCCUTCHAN TF & MILLER LH (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains *Nature* **333** 74-76

32 KAUSHAL DC, CARTER R, RENER J, GROTENDORST CA, MILLER LH & HOWARD RJ (1983) Monoclonal antibodies against surface determinants on gametes of *Plasmodium gallinaceum* block transmission of malaria parasites to mosquitoes *J Immunol* **131** 2557-2561

33 KOCKEN CHM, MILEK RLB, LENSEN THW, KASLOW DC, SCHOENMAKERS JGG & KONINGS RNH (1995) Minimal variation in the transmission-blocking vaccine candidate Pfs48/45 of the human malaria parasite *P falciparum* *Mol Biochem Parasitol* **69** 115-118

34 KUMAR N & CARTER R (1984) Biosynthesis of the target antigens of antibodies blocking transmission of *Plasmodium falciparum* *Mol Biochem Parasit* **13** 333-342

35 LENSEN A PhD thesis In preparation

36 MEIS JFGM, POOL G, VAN GEMERT GJ, LENSEN AHW, PONNUDURAI T & MEUWISSEN JHETH (1989) *Plasmodium falciparum* ookinetes migrate intracellularly through *Anopheles stephensi* midgut epithelium *Parasitol Res* **76** 13-19

37 MEIS JFGM, WISMANS PGP, JAP PHK, LENSEN AHW & PONNUDURAI T (1992) A scanning electron microscopic study of the sporogonic development of *Plasmodium falciparum* in *Anopheles stephensi* *Acta Trop* **50** 227-236

38 MENDIS KN, MUNESINGHE YD, DE SILVA YNY, KERAGALLA I & CARTER R (1987) Malaria transmission- blocking immunity induced by natural infections of *Plasmodium vivax* in humans *Inf Immun* **55** 369-372

39 MENDIS KN & TARGETT GAT (1982) Vaccination to prevent transmission of *P yoelii* malaria *Parasite Immunol* **4** 117-127

40 MENDIS KN & TARGETT GAT (1979) Immunisations against gametes and asexual erythrocytic stages of a rodent malaria parasite *Nature* **277** 389-391

41 MEUWISSEN JHETH & PONNUDURAI T (1986) Some aspects of transmission blocking immunity reviewed In *Memorias do institudo oswoldo cruz*, Rio de Janeiro, 81 Suppl II, 69-75 International Symposium on malaria

42 MEUWISSEN JHETH, PONNUDURAI T AND VERMEULEN AN (1985) Candidate antigens Studies on the development of a *P falciparum* malaria vaccine for the induction of transmission-blocking immunity In *Proceedings of the Asia and Pacific Conference on Malaria* Siddiqui W A (Editor) Hawaii University of Hawaii, pp 133 142

43 MOELANS IMD, MEIS FGM, KOCKEN C, KONINGS RNH & SCHOENMAKERS JGG (1991) A novel protein antigen of malaria parasite *Plasmodium falciparum,* located on the surface of gametes and sporozoites *Mol Biochem Parasitol* **45** 193-204

44 MOTARD A, LANDAU I, NUSSLER A, GRAU G, BACCAM D, MAZIER D AND TARGETT GAT (1993) The role of reactive nitrogen intermediates in modulation of gametocyte infectivity of rodent malaria parasites *Parasite Immunol* **15** 21-26

45 MUNESINGHE YD, MENDIS KN & CARTER R (1986) Anti gamete antibodies block transmission of human vivax malaria to mosquitoes *Parasite Immunol* **8** 231-238

46 NAOTUNNE T DE S, KARUNAWEERA ND, MENDIS KN & CARTER R (1993) Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates *Immunology* **78** 555 562

47 PONNUDURAI T, MEUWISSEN JHETH, LEEUWENBERG ADEM, VERHAVE JP & LENSEN AHW (1982) The production of mature gametocytes of *Plasmodium falciparum* in continuous cultures of different isolates infective to mosquitoes *Trans Roy Soc Trop Med Hyg* **76** 242-250

48 PONNUDURAI T, LENSEN AHW, MEIS JFGM & MEUWISSEN JHETH (1986) Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system *Parasitology* **93** 263-274

49 QUAKYI IA, CARTER R, RENER J, KUMAR N, GOOD MF & MILLER LH (1987) The 230-kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmission blocking antibodies *J Immunol* **139** 4213-4217

50 RAWLINGS DJ & KASLOW DC (1992) A novel 40-kDa membrane-associated EF-hand calcium-binding protein in *Plasmodium falciparum* *J Biol Chem* **267** 3976-3982

51 RENER J, GRAVES PM, CARTER R, WILLIAMS JL & BURKOT TR (1983) Target antigens on transmission blocking immunity on gametes of *Plasmodium falciparum* *J Exp Med* **158** 976-981

52 SCHERF A, CARTER R, PETERSEN P, ALANO P, NELSON C, AIKAWA M, MATTEI D, DASILVA LP & LEECH J (1992) Gene Inactivation of Pf11-1 of Plasmodium-Falciparum by Chromosome Breakage and Healing Identification of a Gametocyte Specific Protein with a Potential Role in Gametogenesis *EMBO Journal* **11** 2293-2301

53 SERGENT E (1963) Latent infection and immunity Some definitions of microbiology and immunology *In Immunity to protozoa* P C C Garnham, A E Pierce and I Roitt, eds Blackwell Scientific Publications, Oxford pp 39-47

54 SHUTE PG & MARYON M (1951) A study of gametocytes in a west african strain of *Plasmodium falciparum* *Trans R Soc Trop Med Hyg* **44** 421 438

55 SINDEN RE, CANNING EU, BRAY RS & SMALLEY ME (1978) Gametocyte and gamete development in *Plasmodium falciparum* *Proc Roy Soc London* **B201** 375-399

56 SINDEN RE & SMALLEY ME (1976) Gametocytes of *Plasmodium falciparum* phagocytosis by leucocytes in vivo and in vitro *Trans Roy Soc Trop Med Hyg* **70** 344-345

57 SINDEN RE (1983) Sexual development of Malarial parasites *Adv Parasitol* **22** 153-216

58 SINDEN RE & CROLL NA (1975) Cytology and kinetics of microgametogenesis and fertilization in *Plasmodium yoelii nigeriensis* *Parasitology* **70** 53-65

59 TARGETT GAT, HARTE PG, EIDA S, ROGERS NC & ONG CSL (1990) *P falciparum* sexual stage antigens immunogenecity and cell mediated resposes *Immunological letters* **25** 77-82

60 VERMEULEN AN, PONNUDURAI T, BECKERS PJA, VERHAVE JP, SMITS MA & MEUWISSEN JHETH (1985) Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission blocking antibodies in the mosquito *J Exp Med* **162** 1460-1476

61 VERMEULEN AN, ROEFFEN WFG, HENDERIK JBJ, PONNUDURAI T, BECKERS PJA & MEUWISSEN JHETH (1985) *Plasmodium falciparum* transmission blocking monoclonal antibodies recognize monovalently expressed epitopes *Dev Biol Stand* **62** 91 97

62 VERMEULEN AN, VAN DEURSEN J, BRAKENHOFF RH LENSEN AHW, PONNUDURAI T & MEUWISSEN JHETH (1986) Characterization of *Plasmodium falciparum* sexual stage antigens and their biosynthesis in synchronized gametocyte cultures *Mol Biochem Parasit* **20** 155 163

63 VOLLER A & BRAY RS (1962) Fluorescent antibody staining as a measure of malarial antibody *Proc Soc Exp Biol Med 110* 907-910

64 WHO World Malaria Situation Weekly epidemiological report (1992) **67** 161-168

65 WILLIAMSON KJ, MD CRISCIO & DC KASLOW (1993) Cloning and Expression of the Gene for *Plasmodium falciparum* Transmission Blocking Target Antigen, Pfs230 Short Communication *Mol Biochem Parasitol* **58** 355-358

66 WILSON DB (1936) Rural hyperendemic malaria in Tanganyika Territory *Trans Roy Soc Trop Med Hyg* **29** 583

67 WINGER LA, TIRAWANCHAI N, NICHOLAS J, CARTER HE, SMITH JE & SINDEN RE (1988) Ookinete antigens of *Plasmodium berghei* Appearance on the zygote surface of an 21 kD determinant identified by transmission blocking monoclonal antibodies *Parasite Immunol* **10** 193 207

68 WIZEL B & KUMAR N (1991) Identification of a continuous and cross-reacting epitope for *Plasmodium falciparum* transmission blocking immunity *Proc Natl Acad Sci USA* **88** 9533-9537

CHAPTER 2

Plasmodium falciparum: A comparison of the activity of Pfs230-specific antibodies in an assay of transmission-blocking immunity and specific competition ELISAs.

W. ROEFFEN, P. BECKERS, K. TEELEN, T. LENSEN, R.W. SAUERWEIN, J.H.E.TH. MEUWISSEN AND W. ELING.

University of Nijmegen; Department of Medical Microbiology, section Parasitology

Published in: *Experimental Parasitology* (1995). 80:15-26

ABSTRACT

The activity was analysed of monoclonal antibodies (mAbs) that specifically recognize the *P.falciparum* sexual stage specific protein Pfs230 All mAbs reacted with the surface of extracellular sexual forms of the parasite in a "suspension immunofluorescence antibody reaction (SIFA)" and precipitated the Pfs230 protein from an NP-40 extract of surface radio-iodinated macrogametes/zygotes. Only mAb that bound complement blocked transmission whereas mAb that did not bind complement but competed with the complement binding mAb for binding to the same epitope did not block transmission. These mAbs were used to develop Pfs230 specific competition ELISAs to analyse epitope diversity and to analyze the binding characteristics of anti-Pfs230 antibodies in human serum. Transmission-blocking (TB) antibodies in test/field sera competed in the competition ELISA for binding with epitope specific, labelled mAbs against Pfs230 At least 5 different epitope regions could be defined with the competition ELISAs

All 46 sera from gametocyte carriers immunoprecipitated the Pfs230 molecule while 19 of these sera blocked transmission in the bioassay. Five of the transmission-blocking and one of the non-blocking sera competed with monoclonal antibodies A method comparison analysis was used to determine agreement between reactions in a competitive ELISA and the TB-activity examined in the bioassay. The index of agreement K between outcomes of the bioassay and ELISA was fair to poor ($K = 0.25$) but since its range include values below 0 the relation between the data obtained by the bioassay and the competition ELISA can be explained by chance alone The serological data did not reveal a correlation between immunoprecipitation of Pfs230 and TB-activity.

INTRODUCTION

Malaria is transmitted from man to mosquito through gametocytes that develop in the blood of infected patients and are taken up by the female mosquito when taking a bloodmeal. Gametocytes of *P.falciparum* synthesize molecules of apparent Mr on SDS-PAGE of 230 kDa and 48/45 kDa (Pfs230 and Pfs48/45), which remain exposed on the surface of macrogametes and zygotes [10,23,25] These molecules and a surface molecule of apparent Mr of 25 kDa (Pfs25) presented on developing zygotes, retort cells and ookinetes are the targets of mAbs some of which can block the subsequent differentiation of the sexual stages in the mosquito; this is defined as TB-immunity [20,21,23] TB-antibodies are found in sera from people exposed to natural malaria and are - at least in part - associated with immunoglobulin reactivity of these sera [13,14].

The conventional bioassay of TB-activity is based on development of oocysts in mosquitoes after membrane feeding of gametocytes as described by Vermeulen *et al.* [23].

It is a costly, labour intensive assay thus limiting the number of sera that can be tested Development of a serological test predicting TB-activity would greatly simplify the study of the significance of TB-immunity A number of studies indicate that antibody responses to epitopes of Pfs48/45 and Pfs230 are part of the response observed after several malaria episodes [4,6,7] and that some epitope regions are conserved in many isolates [5] Immunoprecipitation with sera from people living in endemic areas precipitated radio-iodinated Pfs48/45 and Pfs230 In Papua New Guinea sera there was a good correlation between TB activity and antibody response to ^{125}I labelled Pfs230 although no such correlation was found for the Pfs48/45 antibody reactivity [6] Among the Sri Lanka sera no correlation was found between TB-activity and the presence of antibodies to Pfs230 as detected by immunoprecipitation of radio-iodinated gamete proteins [19]

In the present study a Pfs230 enriched fraction from gametocytes was used for the production of anti Pfs230 mAbs and development of ELISAs Competition ELISAs using different HRPO labelled mAbs, immunoprecipitations and Western blot analysis were developed to characterize antibody reactivity to various epitopes of Pfs230 in sera collected from gametocyte carriers Reactivity in the competition ELISA was compared to TB-activity in the bioassay to analyze a possible predictive value of the competition ELISA for TB-activity

MATERIALS AND METHODS

Parasites

Mature gametocytes of *P falciparum* (isolate NF54) were produced in an automated large-scale culture system as described by Ponnudurai *et al* [16] Mature gametocytes were isolated at 37°C to prevent gametocyte activation as follows Parasite culture was diluted ten fold in M199, containing 0 1% glucose (M199-G) After centrifugation for 15 minutes at 2,000g the pelleted parasites were resuspended in M199-G, loaded on a cushion of 18% Nycodenz and centrifuged for 30 minutes at 5,000g as described by Vermeulen *et al* [23] The purified gametocytes were used a) directly, for SIFA, b) allowed to exflagellate to collect macrogametes and zygotes for use as dried antigen in IFA, or c) stored at -70°C until used for extraction of Pfs230

Macrogametes and zygotes were purified and used for surface radio iodination and immunoprecipitation as described by Vermeulen *et al* [23]

The Pfs230 enriched aqueous phase (AP) extract of gametocytes

Gametocytes suspended in 140 mM NH_4Cl in 10 mM Tris/HCl (pH 7 4) were incubated on ice for 5 minutes to lyse the erythrocyte membrane and parasites were

pelleted by centrifugation (5 minutes, 16,000g at RT) Parasites were solubilized in 2% TX-114 in a 10 mM Tris/HCl buffer (pH 7 4) containing 1 mM PMSF at 4°C as described by Bordier [2] and Kumar [9] The aqueous phase (AP) and detergent phase (DP) obtained by this method were collected separately The AP (Pfs230 enriched antigen) was dialysed against 0 1 M NH_4HCO_3 and freeze-dried in aliquots equivalent to $2x10^7$ gametocytes

Sera

Field sera 46 sera were collected from gametocyte carriers (aged between 6-36 years) recruited at a dispensary in Yaounde, Cameroon [22]

Positive control Serum of a Dutch expatriate who worked in an endemic malaria area in Tanzania for more than 30 years (St1) This serum was positive in all tests used

Negative control A panel of individual serum samples and a pooled sample of these sera (N56) were obtained from Dutch bloodbank donor volunteers, with no previous history of malaria

Complement Freshly obtained AB-blood from Dutch bloodbank donors with no previous malaria exposure was allowed to clot at room temperature for 1 hour and aliquots of the pooled serum were stored at -70°C until required Freshly thawed samples were used as a source of complement

Rabbit α-PLL serum Rabbit anti-poly L lysine serum was a gift from Dr P Rotmans, Laboratory of Parasitology, University of Leiden, The Netherlands

Monoclonal antibodies

The Pfs230 specific mAb 28F1 has been described by Vermeulen *et al* [23,25] MAb 18F25 was produced and characterized as described by Vermeulen *et al* [23] MAbs 11E3, 12F10, 4H2, 12B3 and 14G8 all reacting with Pfs230 were a kind gift of Dr R Carter, Division of Biological Sciences, Edinburgh, England

For the preparation of new mAbs freshly isolated aqueous phase from a TX-114 extraction (AP), equivalent to 2 x 10^7 parasites in 50 μl PBS, was emulsified in 50 μl Freunds Complete Adjuvant and injected intraperitoneally in BALB/c mice The mice were boosted twice with AP in Freunds Incomplete Adjuvant Serum samples were tested by ELISA (see below) and on Western blots with gametocyte proteins Mice were killed three days after the last boost, spleen cells were fused with myeloma cells (P3/X63-Ag8 - 653) and suitable hybridomas were selected as described in more detail by Vermeulen *et al* [23] The supernates were screened for antibody reactivity in a) an IFA using air-dried sexual stage parasites, b) SIFA and c) a one-site ELISA using AP as antigen (see below)

Positive wells were cloned twice by limiting dilution and further tested on Western blots and by immunoprecipitation (see below). Selected clones were further expanded and ascitic fluids were produced. TB-activity of ascitic fluid or its purified IgG preparations was determined in the bioassay (see below).

Purification of monoclonal antibodies

Ascitic fluid was de-fatted with silicon dioxide (Sigma S-5631) as described by Neoh *et al.* [15], the immunoglobulin (Ig) containing supernate was dialysed against 0 1 M NH_4HCO_3 and freeze-dried in bottles or IgG was further purified using a semi-automated FPLC system (Pharmacia) equipped with a Protein-A column (Pharmacia) The IgG preparation was mixed with buffer A (0.1 M Tris/HCl + 1 0 M Na_2SO_4, pH 8.0) loaded on the column at a flow rate of 1 ml/minute and washed with buffer A for 8 minutes Subsequently, buffer B (0 1 M glycine/HCl pH 2.5) was added to obtain a gradient of 5% to 50% B in 15 minutes followed by elution with 100% B for 5 minutes The IgG containing fractions were pooled, dialysed against 0.1 M NH_4HCO_3 and aliquots stored freeze-dried in bottles. All IgG subclasses including IgG1 can be recovered under these conditions

Surface immunofluorescence assay

SIFA was performed with live macrogametes and zygotes All incubations were done at room temperature Briefly, 10^6 mature gametocytes in 50 μl M199-G were incubated with 50 μl FCS for 15 minutes (for activation and exflagellation) A 50 μl antibody test sample was added and incubated for 15 minutes. After washing with PBS, the cells were incubated with 50 μl FITC-conjugated goat anti-mouse IgG (Cappel 55493) diluted in PBS with 0 05% Evans Blue for 10 minutes After two PBS washings, the cells were examined under a cover glass with vaseline-coated edges on a Leitz Ortholux fluorescence microscope (500x magnification)

Transmission-blocking assay

A bioassay, using membrane feeders to feed *A.gambiae* mosquitoes, was used to determine TB-capacity of serum samples [17,18,23] All samples were separately tested in the presence of active or inactivated complement; all measurements were repeated at least once Field sera were diluted threefold and mAbs or IgG preparation of the sera tenfold with negative human serum Twenty mosquitoes per feeder were dissected 7 days after feeding, the number of oocysts on the stomach wall counted and William's mean (adjusted geometric mean (GM)) oocyst number calculated [26] An experiment was considered

successful if at least 90% of the mosquitoes of the controls carried oocysts The adjusted GM was used to determine the reduction activity (R) according to the following equation

$$R = \frac{T_c - T_t}{T_c} \times 100\%$$

where T_t is the geometric mean for the test feeder and T_c is the geometric mean of 3 controls

Because of assay variation only values over 85% were considered to be a positive transmission-blocking result

Immunoprecipitation

Macrogametes and zygotes were surface radio-iodinated by catalysed iodination (isotope ^{125}Iodine), solubilized by NP40 and the extract used for indirect immunoprecipitation as described by Vermeulen *et al* [23]

Labelling of the mAbs with HRPO

Labelling of FPLC purified mAb with HRPO was performed using the periodate method with an input molar HRPO/IgG ratio of 4 according to Wilson and Nakane [28] The labels were dialyzed against PBS, supplemented with thimerosal (0 01%) and FCS (1%) and the samples freeze-dried in bottles and stored at 4°C

Enzyme-linked-immunosorbent assays

I-Detection of Pfs230

a) Microtiterplates were coated with PLL, washed with PBS and incubated with serial dilutions of AP in PBS Subsequently, free PLL binding sites were blocked by incubation with 0 2 mg/ml Rabbit α-poly-L-lysine Ig in PBS for 30 minutes Following three washes with PBS, the wells were incubated with 50 µl HRPO-labelled anti Pfs230 mAb (2 µg/ml) for 2 hours After a washing step, plates were incubated with 60 µl TMB (0 25 mM 3,3',5,5'-tetra-methyl-benzidine, 0 7 mM H_2O_2, 0 1 M sodiumacetate pH 5 5) substrate solution for 20 minutes The enzyme reaction was stopped by adding 60 µl 4 N H_2SO_4 and the optical density (OD) was determined on an ELISA-reader at 450 nm (Titertek Multiskan MCC/340)

b) Two-site ELISAs were carried out as described by Vermeulen *et al* [24], Zavala *et al* [29] and Carter *et al* [3] Microtiterplates were coated with 50 µl of anti Pfs230-mAb (10 µg/ml) in PBS for 30 minutes Plates were washed three times with PBS and saturated with 5% FCS in PBS After incubation with AP extract the wells were incubated with HRPO-labelled anti Pfs230 mAbs, substrate and the absorbance read at 450 nm as above

II-Competition-ELISAs

Pfs230 was captured from AP extract in microtiterplates as described above under Ia or Ib After three washes with PBS, wells were incubated with a mixture of 30 μl test sample and 30 μl HRPO-labelled anti Pfs230 mAb for 2 hours Competition ELISAs were carried out using serial two fold dilutions of unlabelled anti-Pfs230 mAbs (ranging from 31 - 4000 ng/ml) or human sera (ranging from 1/10 1/640) made up in PBS containing 0 1% FCS The plates were washed, incubated with substrate and the absorbance read at 450 nm as above

All incubations were carried out at room temperature The competition titer is defined as the dilution of the test serum that results in the same OD reading as the (competition negative) control serum Sera with a titer $> 1/20$ are considered positive

Comparison of the TB-assay and competition ELISA for sera

The results of competition ELISAs and the bioassay were compared by a statistical test on agreement using kappa as the index of observed agreement [1,12] The index kappa (K) was calculated from the observed and expected (chance distribution) frequencies on the diagonal of the 2x2 table of outcomes of the bioassay and ELISA (see Table IV)

The approximate standard error of K is $SE(K) = \sqrt{\frac{P_o(1-P_o)}{n(1-P_e)^2}}$

where P_o is observed proportional agreement and

P_e the expected proportional agreement by chance

The approximate 95% confidence interval is given by $K \pm 1\ 96\ SE(K)$

The index of agreement K has a maximum 1 00 when agreement is perfect, a value of zero indicates no agreement better than chance Negative values indicate disagreement The guideline by Altman [1] was used to determine the strength of agreement

RESULTS

Detection and preservation of Pfs230 in gametocyte extracts

Pfs230 was found to be exclusively present in the AP of the TX-114 extract which also contained trace amounts of other protein molecules such as 27 kDa, 16 kDa, 48/45 kDa as detected by specific ELISAs and SDS-PAGE analysis (data not shown)

Immunoreactivity of Pfs230 in the freshly prepared AP with mAbs 18F25 and 28F1 as determined by one or two-site ELISA or on Western blot, decreased rapidly during further processing and storage After two cycles of freeze-thawing of a freshly prepared AP only 20% immunoreactivity was left with mAb 18F25 and no immunoreactivity at all with mAb 28F1 Pfs230 detection did not change using protein staining on SDS-PAGE

gels (data not shown). In addition, immunoreactivity of Pfs230 was lost during freeze-drying in PBS (increased salt concentrations generated during freeze-drying). Exposure to 10 times concentrated PBS or 1 M NH_4HCO_3 solution led to an instantaneous loss of immunoreactivity in the one-site ELISA. Immune reactivity was preserved when AP was dialysed against 0.1 M NH_4HCO_3, freeze-dried and stored at 4°C. Pfs230 immunoreactivity appeared to be stable for 6 to 8 weeks when stored *in vacuo* or under N_2-gas and 2 weeks only when stored under air or in solution.

Immunoreactivity of Anti-Pfs230 mAbs and epitope mapping

All mAbs including the newly developed 63F2A2 (IgG2a), 63F3C8 and 63F6D7 (both IgG1) as well as 18F25, 28F1, 4H2, 12B3 and 14G8 (all IgG1) and 11E3 and 12F10 (both IgG2a) reacted specifically with air dried gametocytes and the surface of freshly prepared live intact macrogametes and zygotes. All above mentioned mAbs precipitated Pfs230 of ^{125}I surface radiodinated gametes (data not shown). On Western blots of NP-40 extracts of non-labelled gametocytes, mAbs 63F2A2, 63F6D7, 12F10, 18F25 and 11E3 recognized proteins of apparent Mr 230 kDa and 260 kDa under nonreducing

TABLE I: Epitope recognition of anti-Pfs230 mAbs in the two-site ELISA.

	Pfs230			HRPO labelled second mAb						
	capture mAb	blot	IgG	18F25	11E3	63F6D7	63F2A2	12F10	63F3C8	28F1
1^A	18F25	+	1	-	-	+	+	+	+	+
	11E3	+	2a	-	-	+	+	+	+	+
2	63F6D7	+	1	+	+	-	-	-	+	+
	63F2A2	+	2a	+	+	-	-	-	+	+
	12F10	+	2a	-	-	-	-	-	-	-
3	63F3C8	-	1	+	+	+	+	+	-	+
	4H2	-	1	+	nd	+	+	+	-	+
	12B3	-	1	+	nd	+	+	+	-	+
4	28F1	-	1	+	+	+	+	-	+	-
5	14G8	-	1	+	+	+	+	+	+	+

Note (+) positive reaction; (-) no reaction; nd, not done; blot, immunoreaction on a Western blot; IgG, immunoglobulin subclass; ^A = Epitope region

conditions. The other mAbs (63F3C8, 14G8, 4H2, 12B3 and 28F1) showed no reactivity on Western blot (see Table I).

Checkerboard titrations with various concentrations of labelled mAbs and AP were performed to establish optimal conditions for Pfs230 detection. Such concentrations of antigen and HRPO-labelled mAb were chosen that maximal reduction of the OD was obtained when small amounts of competing antibody were included in the test. Optimal conditions for the competition ELISA were considered to be present with >60% of maximal binding of HRPO-mAb and an OD reading of > 1.0. For mAbs 63F2A2 (0.3 μg/ml) and 63F6D7 (0.5 μg/ml) equal concentrations of labelled mAb and the corresponding non-labelled mAb at the 50% competition value was found. The HRPO-labelling method did not decrease the affinity of most mAbs. Only with mAbs 28F1 (1.0 versus 0.1 μg/ml) and 63F3C8 (2.5 versus 0.25 μg/ml) was considerably less of the non-labelled, corresponding mAb needed for 50% competition, suggesting that the immunoreactivity of these mAbs was substantially reduced after labelling.

TABLE II; Competition of mAbs in the one-site Pfs230 ELISA.

	Comp	**HRPO labelled mAb**						
	mAb	**18F25**	**11E3**	**63F6D7**	**63F2A2**	**12F10**	**63F3C8**	**28F1**
1^	18F25	+	+	-	-	-	-	-
	11E3	+	+	-	-	-	-	-
2	63F6D7	-	-	+	+	+	-	-
	63F2A2	-	-	+	+	+	-	+
	12F10	-	-	+	+	+	-	-
3	63F3C8	-	-	-	-	-	+	-
	4H2	-	-	-	-	-	+	-
	12B3	-	-	-	-	-	+	-
4	28F1	-	-	-	-	-	-	+
5	14G8	-	-	-	-	-	-	-

note. (+) competition between labelled and nonlabelled mAb, (-) no competition between labelled and nonlabelled mAb; ^ = Epitope region

The epitope specificity of each mAb was determined by a) using different capture and labelled mAbs in two-site ELISAs, and b) competition between labelled mAbs and various unlabelled mAbs in one-site competition ELISAs. None of the labelled mAbs

bound to Pfs230 when captured by the corresponding non-labelled mAbs. The results with both systems (Table I + II) suggest the presence of 5 distinct epitopes or epitope regions with two exceptions; a) when 12F10 was used to capture Pfs230 no reaction with any of the labelled mAbs could be found, whereas 12F10 reacted with Pfs230 captured by several other mAbs. This may indicate that coated mAb 12F10 cannot capture Pfs230. b) Competition between mAbs was independent of the use of the mAb in labelled or unlabelled form, except for 63F2A2. In the one-site ELISA, 63F2A2 competed with labelled 28F1, but labelled 63F2A2 did not compete with unlabelled 28F1. This may indicate that 63F2A2 and 28F1 react with the same epitope but with a considerable difference in affinity. A tentative map of the 5 epitope regions is given in figure 1

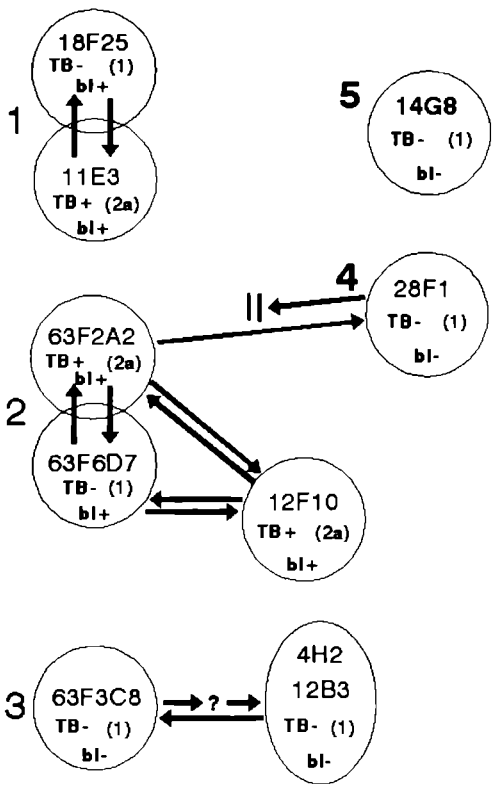


Fig 1 Epitope regions of Pfs230 identified with a series of monoclonal antibodies tested in a one-site competition ELISA MAbs are either effective at blocking transmission of infection to mosquitoes (TB+) or ineffective (TB-) Competitive interactions in the ELISA are indicated as (⟶), no competition (⟶‖) or not tested (⟶?) The isotype of the mAb is given between parenthesis Reaction with Pfs230 on Western blot is given as positive (bl+) and negative (bl-)

Transmission-blocking activity of mAbs

Ascitic fluid and/or purified IgG from the hybridomas were included in the transmission-blocking feeder assay in the presence of either active or inactivated complement Figure 1, shows that 5 μg of mAbs 11E3, 12F10 and 63F2A2 (all IgG2a) blocked transmission more than 99%, but only in the presence of complement. MAbs 18F25, 63F6D7, 63F3C8, 4H2, 12B3, 28F1 and 14G8 (all being IgG1) did not reduce transmission irrespective of the presence of complement Thus, epitope analysis and transmission-blocking experiments show that both blocking and non-blocking mAbs reacted with the same epitope and also compete for binding to that epitope TB-activity correlates with the presence of the complement fixing IgG2a isotypes which are only available for 2 epitopes (see figure 1).

Transmission-blocking activity in human field sera

Forty-six sera from gametocyte carriers, heat inactivated at 56°C for 30 minutes, were tested in feeding experiments in the presence of active and inactivated complement. Nineteen (41.3%) out of these 46 sera consistently blocked transmission in the presence of complement Twelve of the 19 sera with a TB-activity, were again retested in the bioassay both in the presence of either active or inactivated complement. None of these 12 field sera showed a complement dependent transmission-blocking activity (data not shown).

Pfs230-associated TB-activity

All 46 sera of gametocyte carriers precipitated a molecule of apparent Mr on SDS-PAGE of 230 kDa from NP40 extracts of ^{125}I surface radio-iodinated gametes and reacted with molecules of apparent Mr of 230 and 260 kDa on a Western blot of NP40 extracts of non-labelled gametocytes under non reducing conditions (data not shown) These results also showed that the antibodies present in the sera of the gametocyte carriers to ^{125}I labelled Pfs230 precipitated a variable amount of the Pfs230 band. Due to variation of the ^{125}I labelling of various batches of parasites used for these analysis our technique only permitted a semi quantitative analysis and no correlation was found between antibody reactivity to ^{125}I labelled Pfs230 and TB-activity.

These 46 sera were further analysed in a two-site competition ELISA with 18F25 as capture mAb and HRPO-63F2A2 as label Figure 2 shows competition by a serial diluted positive (St1) in comparison to a negative serum (N56). The negative serum did not compete with HRPO-63F2A2 for binding to Pfs230 The overall inter-assay variable in this test (6 times tested in duplicate) has an average deviation of the mean OD at

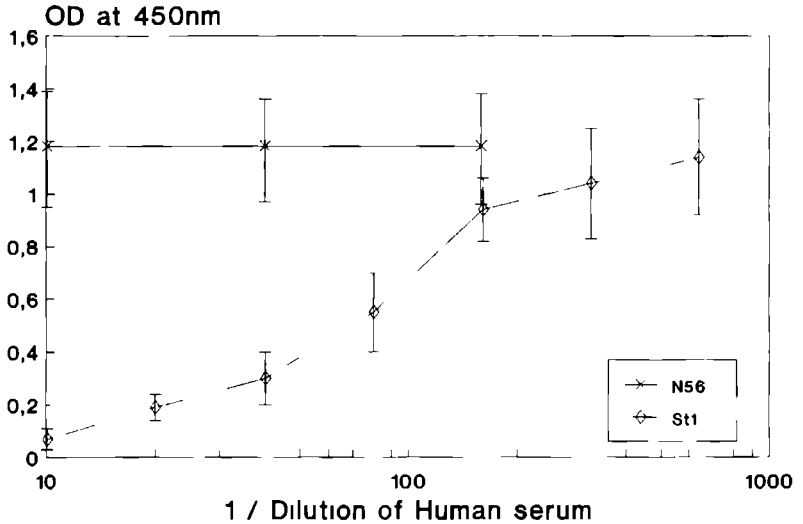


Fig 2 Optical densities at 450 nm with increasing serum dilutions in a two site competition ELISA MAb 18F25 was used to capture the Pfs230 molecule from the aqueous phase and HRPO-63F2A2 for competition with a positive (St1) and negative (N56) human serum

each dilution step of 0 12 (21%) The overall intra-assay variation resulted in an average deviation of the mean OD of 0 09 (12%)

Seven different mAbs reactive to the 5 epitopes/regions of Pfs230 described above including the complement dependent TB mAbs 11E3 and 63F2A2, were labelled with HRPO to investigate their competition with antibodies in serum samples of gametocyte carriers in the two-site competition ELISA Table III shows that competition positive human field sera (6 out of 46) competed with all labelled mAbs i e with all epitopes/regions available, with no exception either in the competition positive or negative sera No significant differentiation in antibody responses to the different Pfs230 epitopes was found Five of these 6 sera blocked transmission Serum 41 (see Table III), the only non-blocking serum reduced transmission in a complement dependent way

The correlation between the TB-activity (positive $\geq 85\%$, negative $< 85\%$) and the Pfs230 competition ELISA is depicted in Table IV Five (26%) serum samples were positive in the TB-assay and in the competition ELISA, 14 (30%) were negative in the

TABLE III, Percentage transmission-reducing activity (R) in the presence of active (AC) or inactivated (IC) complement and the epitope specific competition titer of 5 Pfs230 epitope regions in positive field sera from 46 *P falciparum* gametocyte carriers

	R		HRPO-labelled competing mAb						
Serum	**AC**	**IC**	**18F25 (1)**	**11E3 (1)**	**63F2A2 (2)**	**63F6D7 (2)**	**63F3C8 (3)**	**28F1 (4)**	**14G8 (5)**
4	99	nd[A]	160	320	80	160	80	160	640
19	100	98	80	40	40	80	40	80	320
38	90	nd	80	40	40	160	80	80	nd
39	96	97	80	80	40	160	40	80	320
41	73	18	40	80	40	80	40	40	160
48	89	80	80	80	40	160	40	40	160
N56[B]	0	0	-	-	-	-	-	-	-
St1[C]	100	100	320	160	160	640	80	160	nd

Note ( ) Titer < 1/40, ( ) epitope region, [A] = not done, [B] = a pool from Dutch blood donors with no history of malaria, [C] = a positive control serum which blocks transmission

TABLE IV, Agreement between transmission-blocking activity (TB-assay) and Pfs230 competition ELISA (C230-ELISA) of 46 sera from gametocyte carriers, Cameroon

	No of C230-ELISA serum samples		
TB-assay	**Positive**	**Negative**	**Total**
≥85%	5	14	19
<85%	1	26	27
Total	6	40	46

Relative specificity = 26 of 27, or 96 3%, relative sensitivity = 5 of 19, or 26 3%, Observed proportional agreement (5 + 26)/46 = 0 67, chance proportional agreement (6/46 x 19/46) + (40/46 x 27/46) = 0 57, observed minus chance agreement (0 67 - 0 57) = 0 11, maximum possible agreement beyond chance level (1 - 0 57) = 0 43, agreement quotient (kappa (K)), 0 11/0 43 = 0 25

competition ELISA but positive in the TB-assay, and 26 (56%) were negative in both tests The index of agreement between the outcomes of bioassay and the competition ELISA is K = 0 25, 95% confidence interval -0 06, +0 56 The index indicates (Altman, 1991) that agreement is fair to poor and the negative value in the confidence interval indicates that agreement can be explained by chance The ELISA outcomes show a relative specificity of 96% (26 of 27) and a relative sensitivity of 26% (5 of 19)

DISCUSSION

A panel of monoclonal antibodies belonging to different IgG-subclasses were analysed for Pfs230 binding and TB-activity All mAbs reacted in the indirect IFA with the surface of macrogametes and zygotes of *P falciparum* and all immunoprecipitated Pfs230 from NP40 extracts of surface radio-iodinated macrogametes and zygotes On Western blot prepared from gametocyte extracts, only mAbs 18F25, 11E3, 63F2A2, 63F6D7 and 12F10 recognize Pfs230 under non-reducing conditions In addition, a protein of apparent Mr on SDS-PAGE of 260 kDa is also recognized on blots This difference between results obtained with immunoprecipitation and Western blot analysis has been described before [20] and is best explained by the intracellular localization of a higher Mr precursor of Pfs230 MAbs can also be subdivided by their ability to block transmission, i e all IgG1 mAbs were negative and IgG2a mAbs (11E3, 63F2A2 and 12F10) blocked transmission in a complement dependent reaction Complement dependent lysis of macrogametes and zygotes *in vitro* by Pfs230 mAbs has been described by Quakyi *et al* [20], and has also been found in the *P gallinaceum* system [8]

These mAbs were used for the development of a number of different ELISAs in order to construct an epitope map of Pfs230 (figure 1), to determine the presence of anti Pfs230 antibodies in field sera and to correlate presence of anti-Pfs230 antibodies in field sera with TB-activity of these sera as observed in the bioassay

The Pfs230 antigen used in these ELISAs was prepared by Triton TX-114 gametocyte extraction and phase separation as described by Bordier [2] and Kumar [9] It appeared that immunoreactivity of Pfs230 in these extracts was lost upon storage in PBS solution, after freeze thawing or exposure to high salt concentration The use of aqueous solutions containing a volatile buffer for freeze-drying and storage *in vacuo*, prevented loss of immunoreactivity for periods of 2 months Loss of immunoreactivity of Pfs230 after exposure to high salt concentration has been observed before [11] The loss of immunore activity in solution suggests that oxidative changes could be involved in conformational changes of Pfs230 However, storage of AP in the presence of either vitamin E or glutat hion did not prevent loss of immunoreactivity (data not shown)

One-site ELISAs could not be developed by direct coating of Pfs230 to the plates, but was realized by coating on a precoat of poly L-lysine or Pfs230 specific mAb The positive charge of poly-L-lysine most probably enables binding with the glutamate-rich Pfs230 [27]

This panel of mAbs enabled the recognition of 5 different epitope regions on the Pfs230 molecule, each epitope being present only once Monoclonal antibodies allocated to two epitope regions (here designated 1 and 2) were able to react on a Western blot All mAbs of the complement fixing IgG2a subclass blocked transmission whereas none of the mAbs of the complement non-fixing IgG1 subclass did not Thus, both TB positive and negative antibodies can bind to the same epitope and compete with one another for binding to that epitope MAbs to the three other regions (designated 3 to 5) did not block transmission but these were all of the IgG1 subclass

In a panel of sera from gametocyte carriers from Cameroon all 46 sera immunoprecipitated Pfs230, and 19 sera exhibited TB-activity Sera were classified with respect to their capacity to immunoprecipitate Pfs230 by a semi-quantitative analysis of autoradiograms, compared to the results of the TB-bioassay this revealed no correlation (P-value = 0 396) None out of twelve sera showing TB-activity needed the presence of complement for this activity More sera need to be tested before definite conclusions can be drawn on the role of complement in TB-activity of human field sera

Six sera of these gametocyte carriers from Cameroon were positive in the competition ELISA and this was independent of the epitope region analysed, indicating that there was no epitope restriction Five of these competition positive sera also blocked transmission in the bioassay, but 14 out of the 19 blockers were negative in the competition assays

To compare the outcomes of TB-assay and ELISA a statistical test for comparison of proportions or association is not considered appropriate Besides sensitivity and specificity a measure of agreement is required rather than association The index of agreement kappa (κ) measures the amount of agreement beyond chance and can be used for the correlation analysis In gametocyte carriers the amount of agreement was fair to poor but since the confidence interval contains zero, this implies that the agreement can be explained by chance

In summary, the Cameroon field sera exhibit a) absence of complement dependency of the TB-activity, b) reactivity with all available epitopes of Pfs230 analysed so far and c) no correlation between either the Pfs230 competition ELISA or immunoprecipitation of Pfs230 from NP40 gametocyte extracts and TB-activity as observed in the bioassay This raises the question whether under field conditions TB activity is Pfs230 mediated

Our results are in contrast to results obtained in a series of Papua New Guinea sera,

where Graves *et al.* [6] observed a correlation between immunoprecipitation of Pfs230 and reduction of infectivity to mosquitoes. In our hands all the Cameroon sera precipitated Pfs230 and a semiquatitative analysis on autoradiograms revealed no correlation with TB-activity. One aspect is the difference in study populations. A parallel study analysing TB-activity and antibody reactivity against Pfs230 as determined by immunoprecipitation in a Sri Lanka population failed to show this correlation [19]. Our data do not exclude the possibility that Pfs230 mediated TB-activity might be present in field sera which is complement independent. Another important question is to find out whether Pfs230 mediated TB-activity is always complement dependent.

ACKNOWLEDGEMENTS

We are grateful to Truus Derks and Marianne Sieben for production and purification of monoclonal antibodies, to Marga Bolmer, Arianne Huisman and Geertjan van Gemert for parasite cultures and transmission experiments, to Bert Mulder for supplying the serum samples. The generous gift of various mAbs by Dr R. Carter is thankfully acknowledged. This study was supported by the EEC STJ 004/2, 633.

REFERENCES

1 ALTMAN DG. 1991. Practical statistics for Medical Reseach. Chapman of Hall.

2 BORDIER C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution *Journal of Biological Chemistry* **256**, 1604-1607.

3 CARTER R, BUSHELL G, SAUL A, GRAVES PM & KIDSON C. 1985. Two apparently nonrepeated epitopes on gametes of *Plasmodium falciparum* are targets of transmission blocking antibodies *Infection and Immunity* **50**, 102-106.

4 CARTER R, GRAVES PM, QUAKYI IA & GOOD WF 1989 Restricted or absent immune responses in human populations to *Plasmodium falciparum* gamete antigens that are targets of malaria transmission-blocking antibodies. *Journal of Experimental Medicine* **169**, 135-147

5 FOO A, CARTER R, LAMBROS C, GRAVES P, QUAKYI I, TARGETT G, PONNUDURAI T & LEWIS G 1991. Conserved and variant epitopes of target antigens of transmission-blocking antibodies among isolates of *Plasmodium falciparum* from Malasia. *American Journal of the Tropical Medicine and Hygiene* **44**, 623-631

6 GRAVES PM, CARTER R, BURKOT TR, QUAKYI IA & KUMAR N 1988. Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera *Parasite Immunology* **10**, 209-218

7 GRAVES PM, DOUBROVSKY A, CARTER N & BECKERS P 1990 High frequency of antibody response to *Plasmodium falciparum* gametocyte antigen during acute malaria infections in Papua New Guinea highlanders. *American Journal of Tropical Medicine and Hygiene* **44**, 515-520.

8 KAUSHAL DC, CARTER R, RENER J, GROTENDORST C, MILLER LH & HOWARD JH 1983 Monoclonal antibodies against surface determinants on gametes of *Plasmodium gallinaceum* block transmission of malaria parasites to mosquitoes *Journal of Immunology* **131**, 2557-2562

9 KUMAR N & CARTER R 1984 Biosynthesis of the target antigens of antibodies blocking transmission of *Plasmodium falciparum* *Molecular and Biochemical Parasitology* **13**, 333-342

10 KUMAR N 1985 Phase separation in Triton X-114 of antigens of transmission blocking immunity in *Plasmodium gallinaceum* *Molecular and Biochemical Parasitology* **17**, 343-358

11 KUMAR N & WIZEL B 1992 Further characterization of interactions between gamete surface antigens of *Plasmodium falciparum* *Molecular and Biochemical Parasitology* **53**, 113-120

12 LANDIS JR & KOCH GG 1977 The measurement of observed agreement for categorical data *Biometrics* **33**, 159-174

13 MEUWISSEN JHETH, PONNUDURAI T, VERMEULEN AN & SMITS M 1985 Candidate antigens Studies on the development of a *P falciparum* malaria vaccine for the induction of transmission-blocking immunity In *Proceedings of the Asia and Pacific Conference on Malaria,* Siddiqui W A (Editor) Hawaii University of Hawaii, pp 133 142

14 MULDER B, TCHUINKAM T, DECHERING K, VERHAVE JP, CARNEVALE P, MEUWISSEN JHETH & ROBERT V 1993 Malaria transmission blocking activity in experimental infections of Anopheles gambiae with naturally infected Plasmodium falciparum gametocyte carriers *Transactions of the Royal Society of Tropical Medicine and Hygiene* in press

15 NEOH SH, GORDON C, POTTER A & ZOLA H 1986 The purification of mouse monoclonal antibodies from ascitic fluid *Journal of Immunological Methods* **91**, 231-235

16 PONNUDURAI T, LENSEN AHW, LEEUWENBERG ADEM & MEUWISSEN J HETH 1982 Cultivation of fertile *Plasmodium falciparum* gametocytes in semi-automated systems 1 Static cultures *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76,** 812 818

17 PONNUDURAI T, VAN GEMERT GJA, BENSINK T, LENSEN AHW & MEUWISSEN JHETH 1987 Transmission blockade of *Plasmodium falciparum* its variability with gametocyte numbers and concentration of antibody *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81,** 491-493

18 PONNUDURAI T, LENSEN AHW, VAN GEMERT GJA BENSINK MPE, BOLMER M & MEUWISSEN JHETH 1989 Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes *Parasitology* **98** 165-173

19 PREMAWANSA S, GAMAGE-MENDIS A, PERERA L, BEGARNIE S, MENDIS K & CARTER R 1994 *Plasmodium falciparum* transmission-blocking immunity under conditions of low endemicity as in Sri Lanka *Parasite Immunology* **16**, 35 42

20 QUAKYI IA, CARTER R, RENER J, KUMAR N, GOOD MF & MILLER LH 1987 The 230 kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmission-blocking antibodies *Journal of Immunology* **139,** 4213-4217

21 RENER J, GRAVES PM, CARTER R, WILLIAMS JL & BURKOT TA 1983 Target antigens of transmission blocking immunity on gametes of *Plasmodium falciparum* *Journal of Experimental Medicine* **158,** 976-981

22 TCHUINKAM T, MULDER L, DECHERING K, STOFFELS H, VERHAVE JP, CARNEVALE P, MEUWISSEN JHETH & ROBERT V 1993 Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* in Cameroon Infectivity of gametocytes of naturally infected gametocyte carriers in Cameroon factors influencing the infectivity of mosquitoes *Tropical Medicine and Parasitology* **44**, in press

23 VERMEULEN AN, PONNUDURAI T, BECKERS PJA, VERHAVE JP, SMITS MA & MEUWISSEN JHETH 1985a Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission blocking antibodies in the mosquito *Journal of Experimental Medicine* **162,** 1460-1476

24 VERMEULEN AN, ROEFFEN WFG, HENDERIK JBJ, PONNUDURAI T, BECKERS PJA & MEUWISSEN JHETH 1985b *Plasmodium falciparum* transmission blocking antibodies recognize monovalently expressed epitopes *Developments in Biological Standardization* **62,** 91-97

25 VERMEULEN AN, VAN DEURSEN J, BRAKENHOFF RH, LENSEN THW, PONNUDURAI T & MEUWISSEN JHETH 1986 Characterisation of *Plasmodium falciparum* sexual stage antigens and their biosynthesis in synchronised gametocyte cultures *Molecular and Biochemical Parasitology* **20**, 155-163

26 WILLIAM CB (1937) The use of logarithms in the interpretation of certain entomological problems Annals of Applied Biology 24, 404-14

27 WILLIAMSON KC, CRISCIO MD & KASLOW DC 1993 Cloning and expression of the gene for *Plasmodium falciparum* transmission-blocking target antigen, Pfs230 *Molecular and Biochemical Parasitology* **58**, 355-358

28 WILSON MB & NAKANE PK 1978 Immunofluorescence and Related Staining Techniques *Elsevier, Amsterdam*, 215

29 ZAVALA F, COCHRANE AH, NARDIN EH, NUSSENZWEIG RS & NUSSENZWEIG V 1983 Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes *Journal of Experimental Medicine* **157**,1947-1957

CHAPTER 3

Transmission-blockade of *Plasmodium falciparum* malaria by anti-Pfs230 specific antibodies is isotype dependent.

W. ROEFFEN[1*], F. GEERAEDTS[1], W. ELING[1], P.BECKERS[1], N. KUMAR[2], T. LENSEN[1] & R. SAUERWEIN[1]

[1] *University of Nijmegen, Department of Medical Microbiology, section Parasitology,*
[2] *Johns Hopkins University, School of Hygiene and Public Health, Department of Molecular Microbiology and Immunology, Baltimore, USA*

Published in: *Infection and Immunity* (1995). 63:467-471.

ABSTRACT

Using the parental hybridoma cell line 63F2A2 that produces specific antibodies of isotype IgG1 (63F2A2.1) against Pfs230 we attempted to enrich for hybridoma's synthesis downstream switch variants' IgG2b (63F2A2.2b) and IgG2a (63F2A2.2a) monoclonal antibodies (mAbs). The parental IgG1 did not reduce the *P.falciparum* transmission in a bioassay irrespective of the presence of complement. MAbs 63F2A2.2b and 63F2A2.2a were effective in reducing the infectivity of *P.falciparum* parasites to *A.gambiae* mosquitoes in membrane-feeding experiments. A transmission-reduction of 91% was accomplished by the 63F2A2.2b switch variant and greater than 99% reduction by the 63F2A2.2a switch variant, but only in the presence of active human complement. Subsequently, the transmission reducing effect of mAb 63F2A2.2b or 63F2A2.2a was confirmed *in vitro* by the rapid lysis of newly formed macrogametes/zygotes in the presence of active complement. MAb 63F2A2.1 did not lysed the newly formed macrogametes/zygotes irrespective the presence of complement.

INTRODUCTION

Malaria is transmitted from man to mosquito through gametocytes that develop in the blood of infected patients. Gametogenesis of *P.falciparum* in the *Anopheles* mosquito midgut is accompanied by the emergence of the gametocyte from the red blood cell. From this moment the extracellular parasites become susceptible to immune factors such as antibodies and complement, taken up during the bloodmeal from the vertebrate host [4].

Gametocytes of *P.falciparum* synthesize molecules of apparent M_r of 230 kDa and 48/45 kDa molecules (Pfs230 and Pfs48/45) after extraction with sodium dodecyl sulfate (SDS) and separation on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Both molecules are expressed on the surface of macrogametes/zygotes. Several monoclonal antibodies (mAbs) reactive to these sexual stage molecules block transmission of the parasites from the vertebrate host to the mosquito vector. Some of these mAbs suppress infectivity of malaria gametocytes to mosquitoes in the presence of active human complement [8,16,19,21,22]. To answer the question whether 63F2A2 mediated transmission-blockade is isotype or epitope dependent, a number of isotype switch variants were prepared and selected for differences in their capacity to fix complement.

Downstream isotype switch variants occur spontaneously in hybridoma cell lines with identical binding sites differing only in heavy-chain isotype [10,11]. The frequency of spontaneously occurring isotypic switch variants vary from 10^{-6} to 10^{-5}/cell/generation [10,17] and a sensitive assay is needed to detect the switched Immunoglobulin (Ig) isotype amid a large amount of Ig of the parental isotype. The technique of sequential sublining

combined with screening by isotype specific ELISA has been proven before to be successful [2,11].

In this study the importance of IgG subclass for transmission-blocking activity is further analysed by production and testing of a number of switch variants of anti-Pfs230 specific mAb 63F2A2.

MATERIALS AND METHODS

Parasites and Pfs230 extract

Mature gametocytes of *P.falciparum* (isolate NF54) were produced in an automated large-scale culture system as described by Ponnudurai et al. [12]. Gametocyte isolation and Triton X-114 (TX-114) extraction of the Pfs230 was obtained as previously described [21]. The freeze-dried aqueous phase extract was diluted in phosphate buffered saline (PBS) and used as the antigen in both the competition and the one-site Pfs230 ELISA.

Antibodies

1) The anti-Pfs230 mAbs 18F25, 63F2A2 (63F2A2.1) and 63F3C8 (all isotype IgG1) and anti-Pfs25 mAb 32F81 (isotype IgG1) have been previously described [21,22]. MAb 18F25 was labelled with horseradish peroxidase (18F25-HRPO) according to the periodate method described by Wilson and Nakane [24]. The labelled mAb was dialysed against PBS, thimerosal (0,01%) and fetal calf serum (FCS, 1%) added and samples were stored at 4°C. MAb 32F81 was labelled with fluorescein isothiocyanate (32F81-FITC) following the method described by Goding [5]. Monoclonal rat antibodies against different mouse Ig isotypes (RaAM-isotype) IgG1, IgG2a and IgG2b and HRPO labelled monoclonal rat-anti-mouse kappa light-chain antibodies (RaAM-k-HRPO) were purchased from CLB (Amsterdam, the Netherlands).

2) Complement: AB-blood from Dutch bloodbank donors, with no previous malaria exposure was allowed to clot at room temperature for 1 hour and aliquots of the pooled serum were stored at -70°C until required. Freshly thawed samples were used only ones as a source of complement. The serum was heated at 56°C for 30 minutes to inactivate complement activity. The serum was also used as a malaria negative control serum in the transmission and parasite lysis tests.

Selection, enrichment and purification of isotype switch variants

Selection and enrichment of isotype switch variants were conducted by the technique of sequential sublining [2,11]. Briefly, the parental 63F2A2.1 hybridoma cells were grown in a 96 well microtiter plate (NUNC, Intermed) containing 1,000 cells per well, in Dul-

becco's Modified Eagles Medium supplemented with 20% FCS and 2 5 units interleukin 6 per ml and cultured in a humidified 5% CO_2-in-air-incubator at 37°C After 7 days the culture supernatants were tested in the isotype-specific ELISA and cells from positive wells were redistributed at 50 cells per well After a culture period of 4 days the supernatants were tested, cells of positive wells subcultured at 2 cells per well and cloned by limiting dilution Supernatants were tested for the presence of the parental isotype and/or switch variants

Selected cloned lines were expanded in culture and ascitic fluids were produced from 10^6 hybridoma cells injected in pristane primed BALB/c mice IgG was purified from ascitic fluid using a semi-automatic FPLC system (Pharmacia) equipped with a Protein-A column (Pharmacia) as previously described [21]

Enzyme-Linked Immunosorbent Assay's (ELISAs)

a) The Isotype-specific ELISA was used as previously described by Boot et al [2] The reliable ELISA detection limits were 5 ng/ml for IgG1, IgG2b or IgG2a using standard IgG isotype controls Possible crossreactivity between isotypes was below 0 1%

b) A specific one-site ELISA for detection of antibodies against Pfs230 was carried out as previously described [21]

All incubation steps were done at room temperature except overnight incubations, which were done at 4°C Between the different steps, plates were washed three times using PBS The ELISAs were performed in duplicate and the mean value of the ODs was used for analysis

Transmission-blocking assay

The effect of mAbs on the infectivity of *P falciparum* to *A gambiae* mosquitoes was tested in a bioassay, using membrane feeders as previously described [13,14,22] Briefly while keeping the temperature at 37°C, 14 day old cultures containing fertile gametocytes were mixed with prewarmed uninfected red cells and mAbs, introduced in prewarmed feeders, and fed to *A gambiae* mosquitoes All mAbs were separately tested in the feeder assay in the presence of active or inactivated human complement Fully engorged mosquitoes were separated and held at 26°C Mosquitoes were dissected seven days after feeding, the number of oocysts, in the mosquito midgut, was counted and William's mean (adjusted geometric mean (GM)) oocyst number calculated [23] The adjusted GM was used to determine the reduction activity [21] and values greater than 85% were consistent in subsequent experiments

Suspension Immunofluorescence Assay (SIFA)

Molecules with an apparent M_r on SDS-PAGE of 25 kDa (Pfs25) appear on the surface of macrogametes/zygotes some hours after zygote formation and this was used as a marker of the further development of sexual stages *in vitro* or in the mosquito midgut (feeder assay, see above)

Suspensions of 10^6 mature gametocytes in 100 µl RPMI 1640 medium were mixed with 100 µl negative serum, 30 µl active or inactivated complement serum and 10 µl mAb 63F2A2 parental or the respective switch variants (5 or 25 µg in PBS) and incubated at 27°C for 3 hours Subsequently 25 µl of the suspension containing 10^5 parasites was added to 25 µl 32F81-FITC 1 40 diluted in 0,05% Evansblue in PBS pH 7 2/2% glucose (0 1 M)/0,1% Na_2EDTA (2 7 mM)/0,05% NaAz (PBG) The parasites were incubated for 1 minute, washed with phosphate buffered glucose solution containing 5% bovine serum (PBGB), centrifuged at 16,000 g for 10 seconds, resuspended in 30 µl PBGB, placed in a Burker Turk counting chamber and Pfs25 positive macrogametes/zygotes were counted under a Leitz Ortholux fluorescence microscope (x500 magnification)

Isotype dependent lysis of macrogametes/zygotes

One hundred µl suspension containing 10^6 vital mature gametocytes in RPMI 1640 medium was mixed with 100 µl negative serum and incubated at 27°C for 30 minutes Twenty-five µl of this suspension was incubated with 25 µl 18F25-FITC for 20 minutes, washed with PBGB by centrifugation at 16,000 g for 10 seconds and the pellet was resuspended in 30 µl PBGB To this sample 5 µl (1 µg) of the mAb 63F2A2 switch variant was added together with 4 µl serum as a source of active or inactivated comple ment Parasites were examined and scored as intact or lysed macrogametes/zygotes using both fluorescence and phase contrast light microscopy (x500 magnification)

RESULTS

Isolation and characterisation of switch variants

When seeded at 1,000 cells per well, IgG2b switch variants were detectable from the parental 63F2A2 1 hybridoma after 7 days of culture in 1 2 wells per microtiter plate Further cloning and subcloning resulted in the isolation of a 63F2A2 switch variant of the IgG2b subclass (63F2A2 2b) Using a similar protocol 63F2A2 mAbs of the IgG2a isotype (63F2A2 2a) were obtained from the 63F2A2 2b producing hybridoma All switch vari ants were subjected to limiting dilution conditions to reach purity at clonal level The switch variants reacted only in the ELISA that was specific for the particular isotype (data

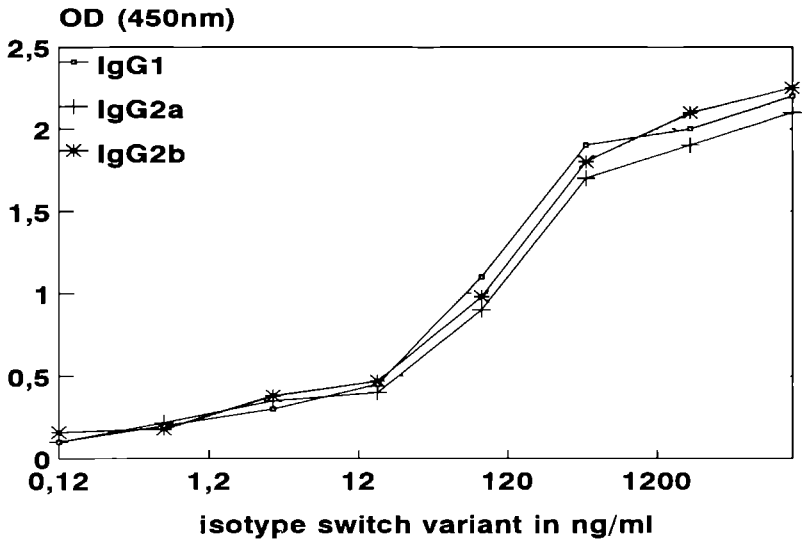


Fig 1 Reactivity of IgG1, IgG2a and IgG2b isotype variants of mAb 63F2A2 in the one-site Pfs230 ELISA Protein-A purified mAbs were serially diluted ranging from 10 μg/ml to 0 12 ng/ml in PTG Results are expressed as the mean of duplicate incubations

not shown) Figure 1 shows similar binding profiles of the parental mAb 63F2A2 1 and its IgG2b and IgG2a switch variants in the Pfs230 one site ELISA

Transmission-blocking capacity of isotypes

The effect of IgG subclass specificity on transmission was analysed in the bioassay in the presence of active or inactivated human complement In the presence of active complement, anti-Pfs230 mAbs 63F2A2 2b or 63F2A2 2a suppressed infectivity of *P falciparum* to mosquitoes by $\geq$90% and $\geq$99% respectively at concentrations of 10 μg/feeder (Table 1) In the presence of inactivated complement the different isotypes of mAb 63F2A2 had no significant effect relative to the mean of the three controls MAb 63F2A2 1 did not reduce oocyst number, irrespective of the presence of complement Oocyst numbers of the control group with active complement were not significantly different from those in the data of the inactivated complement group (paired samples t test t=0 56, degrees of freedom (df)=28, P=0 58)

Table 2 shows dose-dependent reduction of oocyst numbers in the presence of mAb 63F2A2 2b or 63F2A2 2a at concentrations varying from 0 2 to 25 μg/feeder (0 8 to 100

μg/ml). Even at a concentration of 0.2 μg mAb 63F2A2.2a / feeder the geometric mean number of oocysts was 0.9 with a SD of 0.3 in the presence of active complement which result in a reduction activity of 80%. In several independent experiments the reduction capacity of mAb 63F2A2.2b was lower than mAb 63F2A2.2a.

TABLE 1. Effects of different isotypes of anti-Pfs230 mAb 63F2A2 on infectivity of *P.falciparum* gametocytes to *A. gambiae* mosquitoes in membrane feeding experiments with active or inactivated complement.

mAb		Oocyst count[B]							
63F2A2	C[A]	Experiment 1				Experiment 2			
isotype		GMW	SD	R	P/D	GMW	SD	R	P/D
1	+	24.3	5 9	24 5	20/20	2.9	0.9	0	24/24
1	-	20.7	3.6	35.7	20/20	2.7	1.1	5.6	27/30
2b	+	3.2	0.7	90 1	18/20	0.2	0.1	93.9	5/20
2b	-	21.7	4.5	32.6	20/20	2.4	0.8	14 3	14/20
2a	+	0.2	0.1	99.4	4/20	0	0	100	0/20
2a	-	27.2	3.3	25.6	20/20	3.3	1.1	0	24/30
control[C]		32 2	6.5		58/59	2.8	1.4		51/59

[A] = active (+) or inactivated (-) human complement, [B] = The data of experiment 2 are the combined results of two independent tests; [C] = Control is the mean of three feeders per experiment.

GMW, William's mean oocyst number (adjusted geometric mean (GM)); SD, Standard deviation of the GMW; R, Reduction activity according to the equation $R=(T_c - T_t)/T_c$ x 100% where T_t is the GM for the test feeder and T_c is the GM of the three controls; P/D, The number of oocyst positive mosquitoes / number dissected.

Next, twentyfive anti-Pfs230 mAbs of IgG1, 2b or 2a in the form of heat inactivated mouse ascites (final dilution 1:27), provided by Dr R. Carter [18] or developed by one of us (N.K.), were tested for their infectivity of *P.falciparum* gametocytes to mosquitoes. Ten anti-Pfs230 mAbs (isotype IgG2a) and one of isotype IgG2b (mAb 12A1A5) were all effective in blocking the transmission but only when active complement was added to the feeder whereas fourteen IgG1 isotype mAbs did not (Table 3).

TABLE 2. Infectivity of *P.falciparum* gametocytes to mosquitoes of anti-Pfs230 mAb 63F2A2.2b or 63F2A2.2a at various concentrations with active or inactivated complement.

		Oocyst count							
		mAb 63F2A2.2b				mAb 63F2A2.2a			
µg/feeder	C	GMW	SD	R	P/D	GMW	SD	R	P/D
25	+	3.2	0.7	90.1	18/20	0	0	100	0/20
25	-	20.7	3.6	35.7	20/20	4.3	1.1	4.9	18/20
5	+	3.0	0.7	90.7	17/20	0.1	0.1	99.8	2/20
5	-	24.2	4.3	24.5	21/21	3.3	1.0	27.0	16/20
1	+	9.5	2.1	70.5	19/20	0.1	0.1	99.8	2/20
1	-	27.3	6.1	15 2	20/20	4.5	1.3	0 4	17/20
0.2	+				ND	0.9	0.3	80.1	12/20
0.2	-				ND	4 7	1.6	0	16/20
control		32.2	6.5		58/59	4.5	1.3		46/59

See legend of Table 1 for explanation of the different items. ND: not done

TABLE 3. Effect of anti-Pfs230 mAbs in the presence of active complement on infectivity of *P.falciparum* gametocytes (isolate NF54) to *A.gambiae* mosquitoes.

mAb	Isotype	R[A]	Source[B]
5A8, 12C5, 8C10, 9C3, 4H2, 12B3, 14G8, 8D9	IgG1	0 - 24	I
7E10H7, 9F2A10, 14D7, 14H10A2, 13H10E1, 2G8E5	IgG1	0 - 35	II
11E3, 12F10, 1H2, 3G9, 7A6, 8C11, 17E9, 4C10	IgG2a	100	I
12A1A5, 11C12F7, 21C1	IgG2a[C]	100	II

[A] = percentage oocyst reduction in the transmission-blocking assay. See for formula the legend of table 1, footnote B; [B] = Anti-Pfs230 mAbs provided by (I) Dr. R Carter (18) and (II) developed by Dr. N. Kumar; [C] = MAb 12A1A5 has an IgG2b isotype.

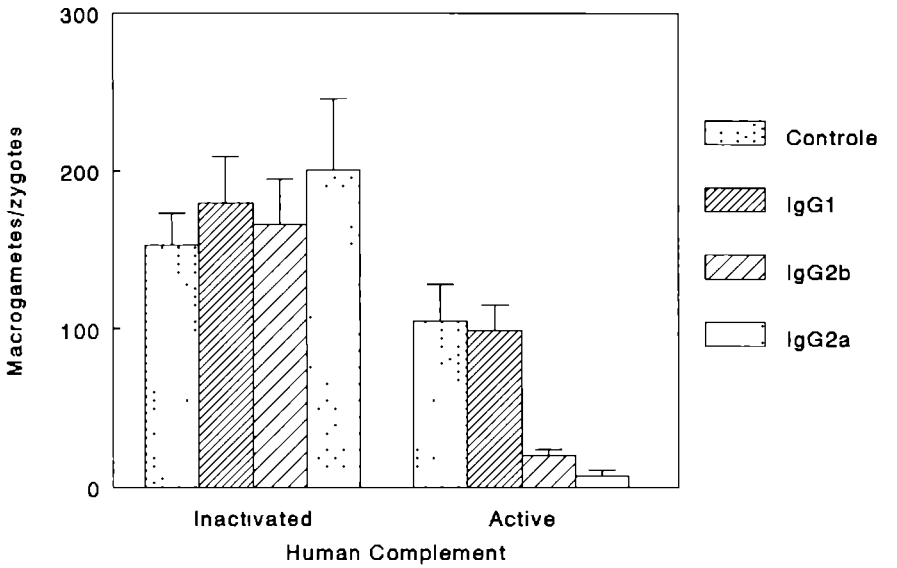


Fig 2. Effect of different isotypes of mAb 63F2A2 on the *in vitro* development of macrogametes/zygotes as detected by the number of Pfs25 positive macrogametes/zygotes per μl.

SIFA and complement-mediated lysis of macrogametes/zygotes

The mechanism of complement-dependent transmission-blocking activity of mAbs 63F2A2.2b and 63F2A2.2a was further analysed *in vitro* with activated gametocytes and a SIFA based on antibody reactivity to Pfs25 thereby monitoring development of macrogametes/zygotes 3 hours after activation. The actual number of macrogametes/zygotes differs in several parasite-batches which makes direct comparison of data of independent experiments impossible. In a representative experiment (Figure 2), no statistical significant differences were found in macrogametes/zygotes numbers between the different isotypes and the controls (no mAb added) when inactivated complement was added (e.g. paired samples t test between IgG2a and control: $t = 1.37$, degrees of freedom (df)=2, $P = 0.305$). In addition, no significant difference was found between the number of macrogametes/zygotes for the control groups (absence of specific anti-Pfs230 mAb) with active complement in comparison to inactivated complement ($t = 4.13$, df=2, $P=0.054$). When the data of the control and the IgG1 group are compared with respect to the effect of complement a significant reduction was observed in the presence of active complement ($t = 4.43$, df=5, $P=0.007$). Active complement in the presence of mAb 63F2A2.1 had no

effect but in the presence of mAb 63F2A2.2b or 63F2A2.2a the number of macrogametes/zygotes was reduced substantially and almost completely in comparison to the effect of the control group (t =6.62, df=2, P=0.022 and t =6.47, df=2, P=0.023 respectively).

Lysis of macrogametes/zygotes by the complement fixing mAb 63F2A2.2a in the presence of active or inactivated complement is shown in fluorescent microscope micrographs using SIFA and the FITC-labelled anti-Pfs230 mAb 18F25 (Fig. 3). In the absence of lysis fluorescence was clearly visible at the circumference of apparently intact parasites (Fig. 3A) whereas fluorescence after lysis either revealed no round forms or damaged round forms with fluorescent debris or fluorescence of incomplete round forms (Fig. 3B). Using phase contrast light microscopy, damaged parasites manifested with an extrusion of the pigment or localization of aggregated pigment at peripheral sites and loss of pigment motility.

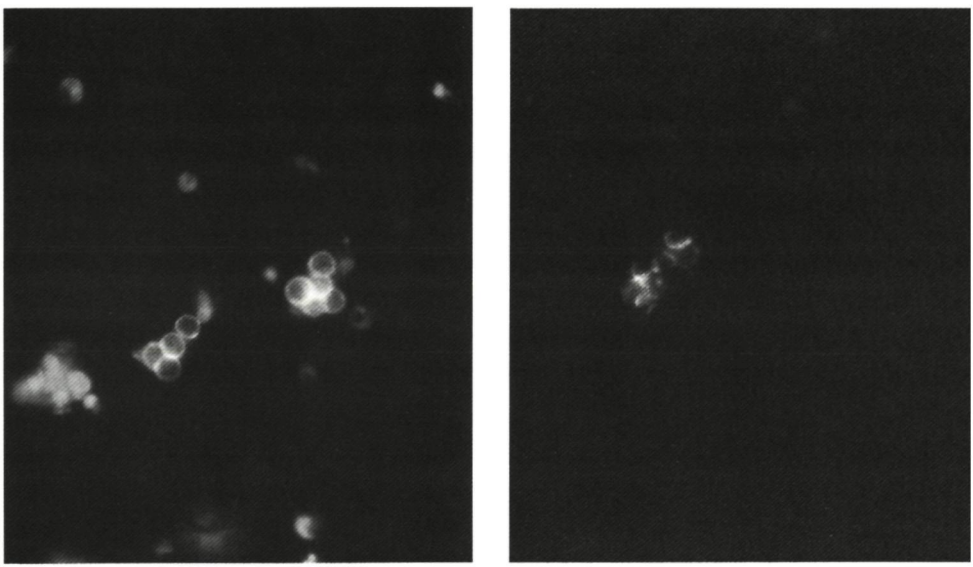


Fig 3. Complement mediated lysis of early *P. falciparum* macrogametes/zygotes in the presence of mAb 63F2A2.2a as observed after staining with mAb 18F25-FITC. The macrogametes/zygotes remained visually intact (A) when incubated with IgG2a switch variant in the presence of inactivated complement and were lysed (B) in the presence of IgG2a switch variant and active human complement.

DISCUSSION

In the present study downstream IgG2b and IgG2a switch variant antibodies specific for Pfs230 were generated from the parent mAb 63F2A2.1. In contrast to 63F2A2.1, both 63F2A2.2b and 63F2A2.2a were able to block the transmission of *P falciparum* to *A gambiae* mosquitoes in membrane feeding experiments but only in the presence of active complement. Results from the one-site Pfs230 ELISA (Fig. 1) show that the dose dependent binding of mAbs 63F2A2.1, 63F2A2.2b and 63F2A2.2a to Pfs230 is comparable. SIFA results suggest a complement dependent disturbance of the sexual development of macrogametes/zygotes after binding of 63F2A2.2b or 63F2A2 2a switch variant mAbs. Even in the presence of active complement the parental mAb 63F2A2.1 did not prevent the development of parasites after activation of the mature gametocytes, however, irrespective the presence of these mAbs, active complement shows a direct effect on macrogametes/zygotes development (Fig. 2) This may be explained by an alternative activation of the complement cascade due to a direct interaction between C3 and the parasites, similar to the stimulation observed with certain bacteria. Although some reduction in the number of viable parasites is accomplished by this activation of the alternative pathway of complement, this does not lead to transmission-blockade Whether this phenomenon applies to other epitopes of Pfs230 [21] remains to be determined

All anti-Pfs230 mAbs of isotype IgG2b or IgG2a tested so far were effective in blocking the transmission but only when complement was added to the feeder whereas IgG1 isotype mAbs did not So far there is an absolute correlation between Pfs230-related transmission-blockade by mAbs, the ability of the isotype to fix complement and the need for active complement in order to show transmission-blockade by Pfs230-binding antibodies Quakyi et al. [16] already described two Pfs230 specific mAbs (both IgG2a) that suppressed infectivity of *P falciparum* to *A freeborni* mosquitoes by a complement mediated lysis of early macrogametes/zygotes. A comparable effect of complement fixing mAbs of the IgG2a isotype against a *P.gallinaceum* protein that is analogous to Pfs230 was described by Kaushal et al. [8] Confirm our observations, Read et al. [18] found in a recent study also the complement dependent suppresion of gametocytes of *P.falciparum* 3D7 to mosquitoes with anti-Pfs230 mAbs of isotype IgG2a.

Generally, mouse IgG1 is known to be a poor binder of human complement factor C1q, whereas IgG2b and IgG2a bind C1q effectively Binding of C1q is the first step in activation of the classical complement cascade, leading to the formation of the membrane attack complex. Mouse IgG2a is more effective than IgG2b in fixation of human complement factors such as C1q, C4b and C3b [3,9] Although complement fixation in binding tests is not directly proportional to complement activation, haemolysis experiments suggest

that the fixation of C3b is directly proportional to activation of C3b and the terminal lytic sequence [3] The capacity of different murine isotypes to fix complement, perfectly fits the ability of mAb 63F2A2 switch variants to interfere with complement

These results may have important implications for the study of anti-Pfs230 antibodies that are present in 40-85% of the endemic human sera [6,20] The presence of anti-Pfs230 specific antibodies in field sera has been associated with transmission-blocking activity by some investigators [6] but could not be confirmed by others [15] or in our study [21] Immunoprecipitation and ELISA [6,21] are based on epitope recognition by antibody being independent of the isotype while a Pfs230 based transmission-blockade may be accomplished by an isotype dependent effector mechanism When Pfs230-components are used in the design of a transmission-blocking vaccine [16], these findings support the need for an immunization strategy [1,7] which should induce antibodies of a complement binding isotype

ACKNOWLEDGEMENTS

We are grateful to Truus Derks and Marianne Sieben for production and purification of monoclonal antibodies, to Marga Bolmer, Arianne Huisman and Geertjan van Gemert for parasite cultures and transmission experiments and JHETh Meuwissen for critically reading and discussion of the manuscript The generous gift of various mAbs by Dr R Carter (Division of Biological Sciences, Edinburgh, England) and Il-6 by Prof L A Aarden (CLB, Amsterdam, Netherland) is gratefully acknowledged This work was supported by EEC STD 004/2, 633 (contract nr TS2-M-0274)

REFERENCES

1 ALLISON AC & NE BYARS 1986 An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity J Immunol Methods **95** 157-168

2 BOOT JHA, MEJ GEERTS, ER DE GROOT & LA AARDEN 1988 Murine monoclonal isotype switch variants Detection with rat monoclonal antibodies in ELISA and isolation by sequential sublining J Immunol Methods **106** 195 202

3 BOOT JHA, PWHI PARREN, MEJ GEERTS, CE HACK & LA AARDEN 1989 Interactions of murine isotype switch variant antibodies with human complement In Immunoglobulin Heavy chain isotype switch variants, page 115 -125' Ph D thesis University of Amsterdam, Amsterdam, the Netherlands

4 CARTER R, N KUMAR, I QUAKYI, M GOOD, K MENDIS, P GRAVES & L MILLER 1988 Immunity to sexual stages of malaria parasites Progress of Allergy **41** 193 214

5 GODING JW 1976 Conjugation of antibodies with fluorochromes modifications to the standard methods J Immunol Methods **13** 215 226

6 GRAVES PM, R CARTER, TR BURKOT, IA QUAKYI & N KUMAR 1988 Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera Parasite Immunol **10** 209-218

7 TEN HAGEN TLM, AJ SULZER, MR KIDD, AA LAL & RH HUNTER 1993 Role of adjuvants in the modulation of antibody isotype, specificity, and induction of protection by whole blood stage *Plasmodium yoelii* vaccines J Immunol **151** 7077 7085

8 KAUSHAL DC, R CARTER, J RENER, CA GROTENDORST, LH MILLER & RJ HOWARD 1983 Monoclonal antibodies against surface determinants on gametes of *Plasmodium gallinaceum* block transmission of malaria parasites to mosquitoes J Immunol **131** 2557-2562

9 LEATHERBARROW RJ & RA DWEK 1984 Binding of complement subcomponent C1q to mouse IgG1, IgG2a and IgG2b a novel C1q binding assay Mol Immunol **21** 321 327

10 MORRISON SL & MD SCHARFF 1981 Mutational events in mouse myeloma cells CRC Crit Rev Immunol **3** 1 22

11 MULLER CE & K RAJEWSKY 1983 Isolation of immunoglobulin class switch variants from hybridoma cell lines secreting anti idiotype antibodies by sequential subliming J Immunol **131** 877-881

12 PONNUDURAI T, AHW LENSEN, ADEM LEEUWENBERG & JHETH MEUWISSEN 1982 Cultivation of fertile *Plasmodium falciparum gametocytes* in semi automated systems 1 Static cultures Trans R Soc Trop Med Hyg **76** 812-818

13 PONNUDURAI T, GJ VAN GEMERT, T BENSINK AHW LENSEN & JHETH MEUWISSEN 1987 Transmission blockade of *Plasmodium falciparum* its variability with gametocyte numbers and concentration of antibody Trans R Soc Trop Med Hyg **81** 491-493

14 PONNUDURAI T, AHW LENSEN, GJA VAN GEMERT MPE BENSINK M BOLMER & JHETH MEU WISSEN 1989 Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes Parasitology **98** 165 173

15 PREMAWANSA S, A GAMAGE MENDIS, L PERERA, S BEGARNIE, K MENDIS & R CARTER 1994 *Plasmodium falciparum* transmission blocking immunity under conditions of low endemicity as in Sri Lanka Parasite Immunol **16** 35 42

16 QUAKYI IA, R CARTER, J RENER, N KUMAR, MF GOOD & LH MILLER 1987 The 230 kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmisssion-blocking antibodies J Immunol **139** 4213-4217

17 RADBRUCH A, B LIESEGANG & K RAJEWSKY (1980) Isolation of variants of mouse myeloma X63 that express changed immunoglobuline class Proc Natl Acad Sci USA **77** 2909 2913

18 READ D AHW LENSEN, S BEGARNIE, S HALEY A RAZA & R CARTER 1994 Transmission blocking antibodies against multiple, non-variant target epitopes of the *Plasmodium falciparum* gamete surface antigen Pfs230 are all complement-fixing Parasite Immunol **16** in press

19 RENER J, PM GRAVES, R CARTER, JL WILLIAMS & TA BURKOT 1983 Target antigens of transmission-blocking immunity on gametes of *Plasmodium falciparum* J Exp Med **158** 976 981

20 RILEY EM, S BENNETT, A JEPSON, M HASSAN-KING, H WHITTLE, O OLERUP & R CARTER 1994 Human antibody responses to Pfs230, a sexual stage-specific surface antigen of *Plasmodium falciparum* non responsiveness is a stable phenotype but does not appear to be genetically regula ted **Parasite Immunol 16** 55-62

21 ROEFFEN W, PJA BECKERS, K TEELEN, T LENSEN, RW SAUERWEIN, JHETH MEUWISSEN & W ELING 1994 *Plasmodium falciparum* A comparison of the activity of Pfs230 specific antibodies in an assay of transmission-blocking immunity and specific competition ELISA Exp Parasitol **79**

22 VERMEULEN AN, T PONNUDURAI, PJA BECKERS, JP VERHAVE, MA SMITS & JHETH MEUWISSEN 1985 Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission blocking antibodies in the mosquito J Exp Med **162** 1460-1476

23 WILLIAM CB 1937 The use of logarithms in the interpretation of certain entomological problems Ann Appl Biol **24** 404-414

24 WILSON MB & NAKANE PK 1978 in Immunofluorescence and related staining techniques Eds Knapp, Holubar and Wick Elsevier/North Holland Biomedical Press, Amsterdam/New York, pp 215-224

CHAPTER 4

Transmission-blocking immunity as observed in a feeder system and serological reactivity to Pfs48/45 and Pfs230 in field sera.

W ROEFFEN, LENSEN, T., MULDER, B, TEELEN, K , SAUERWEIN, R , ELING, W MEUWISSEN, J H E TH AND BECKERS P

University of Nijmegen, Department of Medical Microbiology, section Parasitology

Published in *Mem Inst Oswaldo Cruz, Rio de Janeiro* (1994) 89 Suppl II 13-15

ABSTRACT

Monoclonal antibodies (mAbs) and human sera from gametocyte carriers were applied in the bio-assay to test for their transmission-blocking capacity.
Competition ELISA's have been developed for the detection of natural transmission blocking antibodies. Approximately 55% of the sera blocking in the bio-assay gave positive results in these competition ELISA's.

INTRODUCTION

Gametocytes and macrogametes/zygotes of *P falciparum* synthesize 230 kDa and 48/45 kDa molecules [7]. Several transmission-blocking mAbs had been described which react specifically with these proteins of the sexual stages of *P.falciparum* [3,4,5]. Both surface proteins (Pfs230, Pfs48/45) are also target antigens of natural transmission-blocking (TB) immunity [2]. As their epitopes seem to express a conformational configuration, peptide-constructs cannot be used as immunogens for transmission-blocking antibodies.

The main objective of the present study was to develop a reliable method for the assessment of transmission reducing antibodies in sera of people exposed to malaria infections. Reduced transmission as demonstrated using the laborious bio-assay with *A gambiae* was considered the standard

RESULTS AND DISCUSSION

Solubilizing the antigens from gametocytes to make it suitable for applications in ELISA.

Gametocytes were extracted with Triton X-114 (TX-114) as described by Bordier [1] Phase separation of Triton X-114 extracts results in the separation of Pfs230 in the aqueous phase (AP) and Pfs48/45 in the detergent phase (DP) Immune reactivity of Pfs230 was preserved for at least 8 weeks after freeze-drying and storage under vacuum or under N_2, and Pfs48/45 over one year if stored at -80°C

No Pfs230 was detected with the α-Pfs230 mAbs after directly coating of AP onto the surface of the ELISA-plate. After precoating with poly-L-lysine before coating with AP a one-site ELISA using HRPO-coupled rabbit-anti-mouse conjugate to detect binding of the mAbs could be established. With the DP only a two-site ELISA could be done.

Epitopes of the sexual antigens

In studies using several mAbs with specificity for each of both proteins, it has been established that each protein has more than one epitope, but per molecule of protein each epitope is expressed only once Some of these epitopes interact with blocking others with

non-blocking mAbs as observed in the bio-assay. Also it became evident that the simultaneous presence of more than one of these protein specific antibodies, interacting with the same protein, might cause potentiation of the blocking effect.

MAbs against the 230 kDa protein of hte IgG2a isotype, made by Dr R. Carter, appeared to give a complement dependent blockage by lysis of the macrogametes. The relevance of this phenomenon in sera from endemic areas is presently under study and point to a role for isotypes

In one-site competition ELISA's we have checked all available Pfs230 mAbs against each other At least 5 singly expressed epitopes have been defined on Pfs230. Three are targets of mAbs with no transmission-reducing activity The other two are defined through its reactivity with transmission-reducing and nonreducing mAbs.

The two-site ELISA now appeared utilizable for testing material in epidemiological settings for the testing of specificity to Pfs230 next to that of Pfs48/45.

Pilot study on the relevance of the test for field samples

In view of the epitope results with the mAbs further studies were made with a positive serum, negative sera and a panel of 46 sera taken from gametocyte carriers in an endemic area

The positive serum (St1) collected from a missionary who worked for thirty years in Tanzania, was positive in all assays, blocked transmission in the bio-assay and was used as a reference serum A pool of negative sera (N56) was obtained from Dutch bloodbank donors The gametocyte carriers were recruited at a dispensary in Yaounde, Cameroon

TABLE 1, Comparison of activity in the Pfs230 (C230-ELISA) and Pfs48/45 (C45-ELISA, combined results with 32F3 and 32F1 competition) competition ELISA and transmission reduction activity (TBA) in 46 sera of gametocyte carriers from Cameroon

		TBA		
		≥85% (n=19)	**<85% (n=27)**	**Total**
C230-ELISA	positive	5	1	6
	negative	14	26	40
C45-ELISA	positive	11	2	13
	negative	8	25	33

Six (13%) out of 46 gametocyte carriers were able to compete HRPO-mAbs in the Pfs230 competition ELISA at dilution varying from 1/40 to 1/80.
Eleven (24%) out of 46 gametocyte carriers were able to compete HRPO-32F3 and 8 (17%) sera compete the HRPO-32F1 in the Pfs48/45 competition ELISA at dilution varying from 1/40 to 1/320

Nineteen (41 3%) out of 46 gametocyte sera consistently reduced infectivity in the Nijmegen bio-assay significantly to less than 15% of the control
In table 1 the results are presented of the bioassay (TBA) for transmission reduction (positive $\geq 85\%$, negative $< 85\%$) and competition ELISA for Pfs230 and Pfs48/45 Of the 19 transmission blocking sera 5 (26%) were positive in the C230-ELISA and 11 (58%) in the C45-ELISA (32F3 and/or 32F1 epitope) The Pfs230 test was positive in 6 of the 46 sera, one of these had a TBA score of 73% Two of the 13 positive sera in the Pfs48/45 test had oocyst counts which did not meet the 85% reduction standard There is a positive correlation (for C45-ELISA $X^2=14\ 02$, $P<0\ 001$, for C230-ELISA $X^2=5\ 03$, $P<0\ 04$) between TBA and C45/C230-ELISA, which means if TBA $\geq$ 85% more positives in the C45/C230-ELISA were found In table 2, the results of C230-ELISA and C45-ELISA are compared of the sera with transmission reduction $\geq 85\%$ All 5 sera, positive in the C230-ELISA was also positive in the C45-ELISA Of the 11 sera, positive in the C45-ELISA, 6 were negative in the C230 ELISA

TABLE II, Results of Pfs48/45 (C45-ELISA) and Pfs230 (C230-ELISA) competition ELISA's from 19 sera with high transmission reduction

		C230-ELISA		
		positive	**negative**	**Total**
C45-ELISA	positive	5	6	11
	negative	0	8	8
	Total	5	14	19

In this selection of sera from gametocyte carriers from Cameroon, the Pfs230 specific ELISA's seems to add little or no effect to the transmission-reduction predictive value of the competition ELISA with the Pfs48/45 blocking epitope as target antigen

The use of a capturing antibody for the Pfs230 assay is optional as both mAbs react also with the antigen directly coated to the microtiter plate In the one-site ELISA, the 5 Pfs230 specific mAbs, labelled with HRPO, were added together with serial dilutions of sera in competition for the specific epitope No differentiation in antibody responses to the

Pfs230 epitopes was found, sera positive in one test reacted also with the other epitopes of Pfs230 This is in contrast with the results of our epitope specific Pfs48/45 competition test (C45 ELISA with 32F3 or 32F1 mAb)

The combined competition ELISA's predict about 50% of the transmission reductions higher than 85% Five sera with 100% blockade were absolutely negative in the ELISA's, although the blockade was IgG related

These results might indicate that other Pfs230 epitopes or other *P falciparum* antigens are involved in inducing the production of transmission-reducing antibodies Moreover, transmission-reduction is probably a relatively short lived phenomenon that mainly depends on the temporary presence of gametocytes The incidence of reduction that is prominently present in gametocyte carriers is now being studied in a longitudinal way

ACKNOWLEDGEMENTS

We are grateful to T Derks and M Sieben for production and purification of monoclonal antibodies, to M Bolmer, A Huisman and G J van Gemert for parasite cultures and transmission experiments This study was supported by the EEC STJ 004/2, 633

REFERENCES

1 BORDIER C (1981) Phase separation of integral membrane proteins in Triton X-114 solution *Journal of Biological Chemistry* **256**, 1604-7

2 Graves PM, Carter R, Burkot TR, Quakyi IA & Kumar N (1988) Antibodies to *P falciparum* gamete surface antigens in Papua New Guinea sera *Parasite Immunology* **10**, 209-18

3 QUAKYI IA CARTER R, RENER J, KUMAR N GOOD MF & Miller LH (1987) The 230 kDa gamete surface protein of *P falciparum* is also a target for transmission blocking antibodies *Journal of Immunology* **139**, 4213 17

4 RENER J, GRAVES PM, CARTER R, WILLIAMS JL & BURKOT TA (1983) Target antigens of transmission blocking immunity on gametes of *P falciparum Journal of Experimental Medicine* **158**, 976 81

5 VERMEULEN AN PONNUDURAI T BECKERS PJA VERHAVE JP SMITS MA & MEUWISSEN JHETH (1985a) Sequential expression of antigens on sexual stages of *P falciparum* accessible to transmission blocking antibodies in the mosquito *Journal of Experimental Medicine* **162**, 1460 76

6 VERMEULEN AN, ROEFFEN WFG, HENDERIK JBJ, PONNUDURAI T, BECKERS PJA & MEUWISSEN JHETH (1985b) *P falciparum* transmission blocking antibodies recognize monovalently expressed epitopes *Developments in Biological Standardization* **62**, 91-7

7 Vermeulen A Deursen Van J Brakenhoff RH Lensen THW, Ponnudurai T & Meuwissen JHETh (1986) Characterisation of *P falciparum* sexual stage antigens and their biosynthesis in synchronised gametocyte cultures *Molecular and Biochemical Parasitology* **20** 155 63

CHAPTER 5

A comparison of transmission-blocking activity with reactivity in a *Plasmodium falciparum* 48/45-kD molecule-specific competition ELISA.

W ROEFFEN, T LENSEN, B MULDER, K TEELEN, R SAUERWEIN, J VAN DRUTEN, W ELING, JHETH MEUWISSEN AND P BECKERS

University of Nijmegen, Department of Medical Microbiology, section Parasitology and Department of Medical Statistics

Published in *American Journal of the Tropical Medicin and Hygine* (1995) 52 60-65

ABSTRACT

Monoclonal antibodies (mAbs) 32F1 and 32F3 react with two independent epitopes of a protein doublet with molecular weights 48 and 45 kDa expressed on the surface of *P falciparum* (Pfs48/45) macrogametes and zygotes of which only 32F3 blocks transmissi on These mAbs were used to develop a Pfs48/45 specific competition ELISA using 32F1 to capture antigen and labelled 32F3 for quantification and analysis of the contribution to transmission-blocking (TB) activity of antibodies in human serum

A comparison analysis was used to determine agreement of competition ELISA titers and TB-activity as observed in the bioassay in three groups of serum samples 37 European travellers with previous exposure to malaria, 56 gametocyte carriers and 66 school children from a malaria endemic area in Cameroon The index of agreement K between outcomes of the ELISA and TB assay in gametocyte carriers and in travellers was fair/moderate, in school children the agreement was not beyond chance The combined analysis of all sera showed a significant and fair/moderate agreement between the results of the competition ELISA and the TB assay with a relative specificity of 94% and a relative sensitivity of 44% This study shows that a positive C48/45-ELISA is indicative for TB-activity in the mosquito-assay, while a negative result does not exclude TB activity

INTRODUCTION

Gametocytes of *P falciparum* synthesize 230 kDa and 48/45 kDa molecules which remain exposed on the surface of macrogametes and zygotes [1,2,3] Several mAbs directed against these molecules block transmission of the parasite to the mosquito vector One of the targets of TB-mAbs is the 48/45 kDa glycoprotein doublet (Pfs48/45) Two independent, non repetitive epitopes of this protein react respectively with mAbs designated 32F1 and 32F3 The presence of 32F3 in a mosquito blood meal blocks transmission of the NF54 isolate of *P falciparum* while 32F1 does not [4] The conventional assay measuring transmission-reduction in mosquitoes using a feeder system as described by Vermeulen and others [1] is costly, labour intensive and thus limits the number of sera that can be tested Development of a serologic test predicting TB activity would greatly simplify epidemiological studies A positive correlation between antibody reactivity to Pfs230 and TB-activity has been found using immunoprecipitation to quantify the antibody response Such a relation was not found for antibody reactivity to Pfs48/45 [5]

This paper describes the development of a two-site competition ELISA based on the 32F3 epitope This competition ELISA was used to analyse several sets of field sera In addition, the TB-activity of these field sera was determined using the feeder assay [1] and

the results obtained compared with those of the competition ELISA using a statistical analysis for comparison of methods [6,7]

MATERIALS AND METHODS

Parasites and Pfs48/45 extract

Mature gametocytes of *P falciparum* (isolate NF54) were produced in an automated large-scale culture system as described by Ponnudurai and others [8] Gametogenesis was induced by incubating the mature gametocytes in fetal calf serum (FCS) at room temperature for 30 minutes Macrogametes and zygotes were purified as described by Vermeulen and others [1] Pelleted parasites were stored at -70°C until use Pfs48/45 for use in sandwich competition ELISA experiments, was extracted in 25 mM Tris HCl (pH 8 0), 0 5% Nonidet P40 (NP40), 1 mM phenylmethylsulphonyl fluoride and 1 μg/ml each of DNase and RNase Insoluble debris was pelleted by centrifugation (16,000 g, 5 min at RT) and the soluble protein extract was stored at -70°C The presence and quantity of Pfs48/45 in the extract was determined in a sandwich ELISA using labelled-32F3 and 32F1

Transmission-blocking assay

A bioassay with membrane feeders to feed mosquitoes was used to determine TB-activity [1,9,10] Briefly, while keeping the temperature at 37°C, fourteen day cultures, containing fertile gametocytes were mixed with prewarmed uninfected red blood cells and samples of human sera or mAbs, introduced in prewarmed feeders and fed to *A gambiae* mosquitoes All samples were tested in two experiments in the presence of complement The minimal obtainable dilution of the sera was threefold and the IgG preparations of blocking sera were retested using a tenfold dilution with the malaria negative control serum Fully engorged mosquitoes were separated and held at 26°C Seven days later 20 mosquitoes per feeder were dissected and the number of oocysts on the stomach wall was counted and William's mean (adjusted geometric mean) oocysts number was calculated [11] An experiment was deemed to have succeeded when at least 90% of the mosquitoes examined carried oocysts in each of three controls The adjusted geometric mean was used to determine the reduction activity (R) according to the equation

$$R = \frac{T_c - T_t}{T_c} \times 100\%$$

Where T_t is the geometric mean for the test feeder and T_c is the mean of the three controls

If the transmission reduction value of a given serum was lower than 85% a considerable

variation could be observed among independent repeated tests Values over 85% were consistent in subsequent experiments and only those were considered as positive in the TB-assay in this paper

Reduction of transmission in the feeder assay was confirmed using the immunoglobulin fraction of these sera

Antibodies

Serum samples were obtained from the following groups

1) 56 gametocyte carriers (aged between 6-36 years) recruited at a dispensary in Yaounde, Cameroon [12],
2) 37 European travellers (aged between 6-70 years) to malarious regions with antibody titers to *P fieldi* asexual parasites determined by the indirect immunofluorescence assay (IFA),
3) 66 school children (aged between 8-12 years) from Edea, Cameroon [13]

A panel of individual and pooled sera from Dutch bloodbank donors, without previous malaria experience, was used as negative controls

As a positive control in every test the serum from one individual who had worked in a malaria area in Tanzania for more than 30 years was included

Complement Freshly obtained AB blood from Dutch bloodbank donors, without previous malaria experience, was allowed to clot at room temperature for 1 hour Aliquots of the resulting serum were stored at - 70^{0}C until used

Sandwich Competition ELISA (C48/45-ELISA)

Biotin labelling of mAb 32F3 was performed according to the procedure described by Verhave and others [14], and horseradish peroxidase (HRPO) labelling following the periodate method using an input HRPO/IgG molar ratio of four was carried out according to the method described by Wilson and Nakane [15] The labelled mAbs were dialyzed against phosphate buffered saline (PBS), supplemented with thimerosal (0 01%) and FCS (1%) and stored at 4^{0}C

The basic procedure was that of the two-site ELISA All incubations were carried out at room temperature To capture Pfs48/45, wells of microtiter plates (Hycult) were coated with 50 μl of a 10 μg/ml concentration of 32F1 in PBS for 45 min Plates were washed three times with PBS Wells were saturated with 150 μl of 2% BSA in PBS for 30 min After three washes with PBS, the wells were incubated for 2 hrs with 50 μl of the NP40 parasite extract (see above) containing the equivalent of 200,000 gametes/zygotes diluted in TNP (25 mM Tris-HCl pH 8 0, 150 mM NaCl, 0 1% NP40) Following three washes

with PBS, wells were incubated with 30 μl test serum sample and 30 μl labelled-32F3 for 2 hrs. For the biotin label, an additional incubation step with streptavidin-biotinylated peroxidase complex (Amersham; 1:3,000 diluted in PBS containing 0.05% Tween-20) preceded the incubation with substrate solution. For both labels the wells were washed four times with PBS and incubated for 20 minutes with 60 μl TMB (0.25 mM 3,3',5,5'-tetra-methyl-benzidine, 0.7 mM H_2O_2 in 0.1 M sodium-acetate pH 5.5) substrate solution. The peroxidase-substrate reaction was stopped by adding 60 μl 2 M H_2SO_4 and the optical density (OD) was determined in an ELISA-reader at 450 nm (Titertek Multiskan MCC/-340).

For optimal immunoreaction labelled-mAbs and antigen were tested in a chequerboard titration. From this antigen-antibody titration concentrations of labelled mAb and antigen were chosen resulting in >60% of maximal OD of labelled mAb and an OD reading of >1.0.

Competition is detected as a reduction in the OD reading obtained with the mAb alone in the presence of a negative control serum. The true endpoint is defined as the dilution (titer) at which the positive sample reaches the OD value of the negative control. Sera with true endpoint $\geq$1/20 are considered positive.

Comparison of the TB-assay and competition ELISA

The results of the competition ELISA were compared with those obtained by the TB-assay and analysed by a statistical test on agreement using kappa as the index of observed agreement [6,7]. The index kappa (K) is calculated from the observed and expected (by chance) frequencies on the diagonal of the 2x2 table of TB-activity outcomes in the bioassay ($\geq$85%, <85%) and results of the competition ELISA (positive, negative).
The index is given by $K = (P_o - P_c)/(1 - P_e)$

where 1) P_o = observed proportional agreement, i.e., $P_o = \sum_{i=1}^{n} f_{ii} / n$

were f_{ii} is the number of agreement for category i (i=1,2)
and n is the number of examined sera,

2) P_e = expected proportional agreement by chance, i.e, $P_e = \sum_{i=1}^{2} r_i c_i / n^2$

were r_i and c_i are the row and column totals for the i^{th} category (i=1,2).

The approximate standard error of K is $SE(K) = \sqrt{\frac{P_o(1-P_o)}{n(1-P_e)^2}}$

and the approximate 95% confidence interval is given by $K \pm 1.96\ SE(K)$.

TABLE 1 Guideline for the interpretation of the index of agreement kappa (K).

Value of K	Strength of agreement
≤0 20	poor
0 21 - 0 40	fair
0 41 - 0 60	moderate
0 61 - 0 80	good
0 81 1 00	very good

The index of agreement K has a maximum 1.00 when agreement is perfect, values of zero or below indicate no agreement better than chance. While no absolute interpretation is possible the guideline proposed by Altman [6] was used to determine the strength of agreement (Table 1)

RESULTS

Testing of the labels and competition with serum antibodies

The optimal concentration of labelled-mAb and the optimal amount of antigen coated per well as determined in the chequerboard titrations, were respectively 2 μg/ml for the biotin label and 200,000 macrogamete/zygote-equivalents / well, and 1 μg/ml for the HRPO-label and 100,000 macrogamete/zygote-equivalents / well. The maximal OD of the HRPO-32F3 label was higher than that of the biotin-32F3 label using the same concentration of antigen. Competition of labelled mAb with increasing concentrations of nonlabelled mAb is depicted in Figure 1. The 50% competition values of the corresponding unlabelled mAb were 0 25 μg/ml for the 1 0 μg/ml HRPO-label and 1.3 μg/ml for the 2 0 μg/ml biotin-label. In addition, it appeared that the biotin-32F3 competition ELISA was positive with 7 out of 19 sera having positive TB-activity as observed in the bioassay, whereas the HRPO-32F3 competition was positive for 9 out of 19 sera Due to the limited sensitivity of the biotin-32F3 competition ELISA human sera could not be diluted more than 1 in 10 preventing a complete titration of the sera The mAb 32F1 (anti Pfs48/45) and mAbs against 25 kDa and 230 kDa antigens were negative in the competition test up to a concentration of 330 μg/ml

Figure 2 shows examples of the competition of serial dilutions of sera with the HRPO-32F3 label Data of the negative serum are the mean of 14 individual sera tested

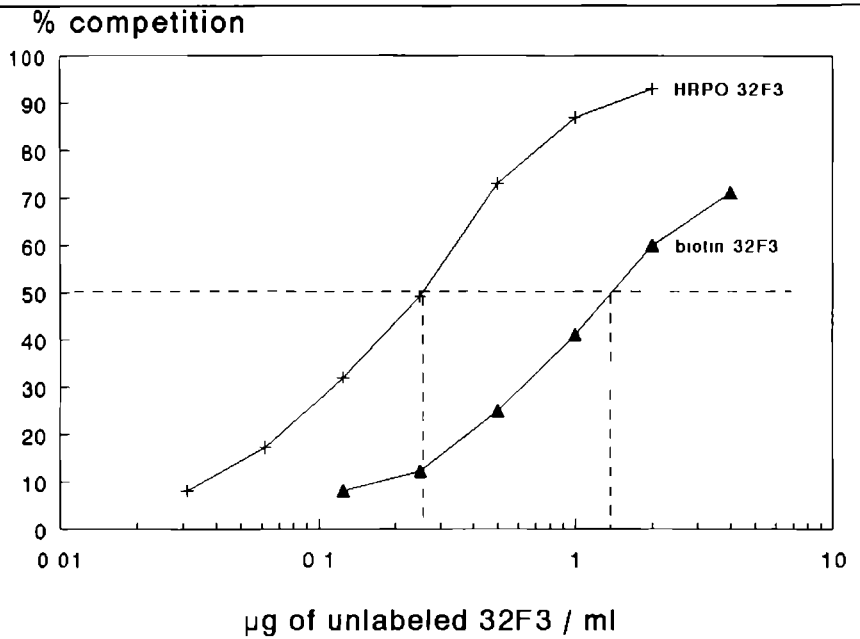


Figure 1 Two-site competition ELISA 32F1 was used to capture Pfs48/45 from a NP-40 extract (+) HRPO-32F3 (1 µg/ml with 100,000 macrogamete/zygote-equivalents/well) and (▲) biotin-32F3 (2 µg/ml with 200,000 macrogamete/ zygote-equivalents/well) was used as label in competition with serial dilutions of nonlabelled 32F3

on 3 separate occasions Calculation of the overall intra-assay variation resulted in an average deviation of the mean OD of 0 08 (10%) The overall inter-assay variation resulted in an average deviation of the mean OD of 0 11 (14%) The titration values of 3 sera that were positive in the competition ELISA are depicted using the overall mean of duplicate determinations of two independent tests

Control experiments with 14 sera from Dutch bloodbank donors exhibited a nonspecific reduction up to a maximum of 15% of the OD of the HRPO-32F3 label compared to reactions without competing serum antibodies This nonspecific reduction was not seen with IgG preparations of these sera Nevertheless, the competition titer found for sera and their corresponding IgG preparations were the same

Comparison of competition ELISA and TB-assay in several groups of sera

Table 2 shows the results of the competition ELISA and the TB assay for the sera of gametocyte carriers, travellers and school children Using the HRPO-32F3 label 17 (30%)

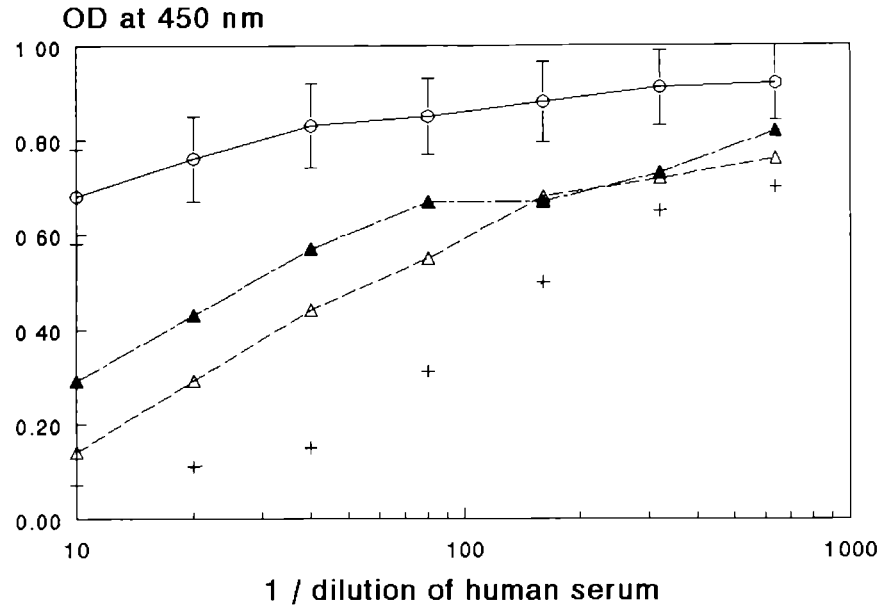


Figure 2. Inhibition of binding of HRPO-32F3 in the presence of serial dilutions of human serum. Negative serum (o); each data point represents the mean ± SD of fourteen determinations performed on three different days. Three sera positive in the competition-ELISA (▲,△,+), each data point represents the mean of duplicate determinations performed on two days. For concentrations of antigen and HRPO-32F3 see legend to Figure 1.

out of 56 of the gametocyte carriers exhibited significant competition in the ELISA. These results were compared with the corresponding TB-activity observed in the bioassay.

By shifting the TB-assay cut-off point in the gametocyte carrier group in the range 10%-90%, it appeared that the maximum degree of agreement between TB-assay and C48/45-ELISA was at the 85% reduction point. Furthermore it appeared that the agreement between TB-assay and C48/45-ELISA was not above chance if the cut-off point of the TB-assay was set at a reduction lower than 70% (95% confidence interval of K included the value 0). At the 85% reduction point the index K assumed the value 0.40, indicating fair/moderate agreement between the outcomes of competition ELISA and TB-assay (Table 3). Four of the 17 positive sera from gametocyte carriers in the C48/45-ELISA test had oocyst counts that did not meet the 85% reduction standard. Of the 25 sera that were positive in the TB-assay, 13 had antibodies competing with the HRPO-

TABLE 2 Activity in the Pfs48/45 competition ELISA (C48/45-ELISA) and in the transmission-blocking assay (TB-assay) in sera of gametocyte carriers, travellers and school children

	C48/45-ELISA test results					
	56 gametocyte carriers		**37 travellers**		**66 school children**	
TB-assay	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**
≥ 85%	13	12	9	10	2	8
< 85%	4	27	0	18	2	54
	17	39	9	28	4	62

TABLE 3 Comparison of activity by index of agreement K in the Pfs48/45 competition ELISA (C48/45-ELISA) and the transmission-blocking assay in sera of gametocyte carriers, travellers and school children

	Index of agreement K		
group	K	**95% conf. interval**	**Strength**
56 gametocyte carriers	0 40	0 15 - 0 65	fair/moderate
37 travellers	0 47	0 19 - 0 75	fair/moderate
66 school children	0 22	-0 23 0 67	not beyond chance

32F3, indicating a relative sensitivity of 52% Of 31 sera considered negative by TB assay, 27 were also found negative by C48/45-ELISA, showing a relative specificity of 87% of the competition ELISA

Nine (24%) out of 37 sera of the group of travellers exhibited significant activity in the ELISA Nine (47%) out of 19 sera, positive in the TB assay had antibodies competing with the HRPO-32F3 All sera (18) considered negative by the TB assay were also negative by C48/45 ELISA The index of agreement K between TB-assay and C48/45-ELISA for the group of travellers, assumed a value of 0 47, indicating moderate agreement (Table 3)

Four (6%) out of 66 sera of school children showed significant competition in the ELISA Only 2 (20%) out of 10 sera, positive in the TB-assay, were positive in the competition ELISA

The relative specificity was 96% (54 out of 56) The index of agreement K between the results of the C48/45-ELISA and the TB-assay was low with a wide confidence interval (K = 0 22, 95% confidence interval -0 23, 0 67) Since the confidence interval contains zero, chance can explain the observed level of agreement

A summary of the data of the 3 groups combined is given in Table 4 The index of agreement K between the results of the C48/45-ELISA and the TB-assay is K = 0 44, with 95% confidence interval 0 28, 0 60 indicating fair/moderate agreement (Table 1) The ELISA outcomes show a relative specificity of 94% (99 of 105) and a relative sensitivity of 44% (24 of 54)

TABLE 4 Comparison# of activity in the transmission-blocking assay (TB-assay) and in the Pfs48/45 competition ELISA (C48/45-ELISA) in all field sera combined

	C48/45-ELISA test results		
TB-assay	**positive**	**negative**	**Total**
≥ 85%	24	30	54
< 85%	6	99	105
Total	30	129	159

Relative specificity = 94% (99 of 105) Relative sensitivity 44% (24 of 54), Index of agreement K = 0 44

DISCUSSION

A mAb based competition ELISA was developed to study the natural antibody reactivity to Pfs48/45 in field sera in comparison to TB-activity as observed in a bioassay The 32F3 labelled mAb was used in this competition ELISA because of its blocking activity in the TB-assay Competition of antibodies in field sera with labelled 32F3 for binding to Pfs48/45 might therefore be relevant for TB-activity Transmission blockade has not been found in serum of rabbits immunized with recombinant Pfs48/45 (unpublished data) Therefor Pfs48/45 extract from parasites was used as antigen in the competition ELISA

The concentration of non labelled antibody necessary to reduce binding of the labelled mAb in the competition ELISA was lower for HRPO-labelled 32F3 than for biotin labelled 32F3 This was supported by the observation that in the competition ELISA with HRPO 32F3 as label 9 out of 19 blocking field sera were positive in contrast to 7 out of 19 when biotin 32F3 was used

Therefore, HRPO-labelled 32F3 was used in the competition ELISA's of subsequent experiments.

TB-activity was found in a high proportion of sera from gametocyte carriers and travellers to malarious regions. In the group of travellers, all sera positive in the C48/45-ELISA blocked transmission, but 10 out of 19 blockers were negative in the C48/45-ELISA. Pfs48/45 has several, surface exposed domains recognised by mAbs [16,17]. Since only one domain potentially involved in blocking activity could be analysed in this study, reactivity to other domains might explain why 10 out of 19 blockers were negative in the C48/45-ELISA. Since sera from people living in endemic areas react to all epitopes tested on the Pfs230 (unpublished data), several alternative explanations may be considered. It may be that blocking reactivity is targeted to other than the Pfs48/45 molecule, or blocking does not only depend on antibody, but may also be caused by nonspecific factors as observed in *P.vivax* and *P.falciparum* infections [18]. In our analyses it appeared, however, that IgG fractions of sera with TB-activity blocked transmission too. Although this does not exclude the presence of nonspecific TB-activity, specific activity can explain the results. It is also possible that the C48/45-ELISA is less sensitive than the TB-assay.

The results of the gametocyte carriers and school children gave an additional problem because respectively 4 out of 17 and 2 out of 4 sera positive in the C48/45-ELISA were in the non-blocking group ($<85\%$) It should be noted that the category R $< 85\%$ in the TB-assay does not necessarily mean that there is no transmission-reduction. It only shows that repeated experiments with sera from this group exhibited considerable variation of the transmission-reduction value, making it impossible to subdivide the group with R value's $< 85\%$ in different subclasses.

A statistical test for comparison of proportions or association is not considered appropriate for the comparison of the outcomes of TB-assay and C48/45-ELISA Besides sensitivity and specificity, measure of agreement is required rather than association. The kappa (K) measure, which measures the amount of agreement beyond chance seems to fit our purpose In 2 of 3 groups of sera agreement was significant, (fair/moderate for gametocyte carriers and travellers) and only in the group of school children agreement between the outcomes of the two methods could be explained by chance Nevertheless, overall a fair/moderate agreement was found. In addition, the C48/45-ELISA exhibited reasonable levels of sensitivity ($\geq 44\%$) and specificity ($\geq 94\%$).

Analyses of a panel of sera from Papua New Guinea using immunoprecipitation of Pfs48/45 and competition of these sera with mAb IIC5B10 did not reveal a correlation between TB-activity and serologic reactivity to Pfs48/45 [5] It may be considered that the TB-activity of IIC5B10 is comparatively low compared to 32F3 [19].

On the other hand, the analyses of the Papua New Guinea sera revealed a correlation between serologic reactivity to Pfs230 and not Pfs48/45 and TB-activity [5]. Our competition is specific (94%) but only positive in 44% of the sera with TB-activity. This leaves the possibility that the competition ELISA is insensitive or that TB-activity in these sera is directed to other sexual stage molecules e g Pfs230 as observed by Graves and others [5]

In summary, the TB-bioassay is a costly, comparatively labour intensive and time consuming

test A Pfs48/45 epitope specific competition ELISA has a significant predictive value to the TB-activity being limited by the fact that only 44% of all TB-sera are positive in the competition ELISA This indicate the need to increase sensitivity of the test and/or to define additional transmission-blocking targets

ACKNOWLEDGMENTS:

We are grateful to Truus Derks and Marianne Sieben for the production and purification of monoclonal antibodies, to Theo Arens and Tita Oettinger for serologic testing, Marga Bolmer, Arianne Huisman and Geertjan van Gemert for parasite cultures and transmission experiments and Christina Celluzzi for critical reading of the manuscript

Financial support This study was supported by the Dutch Ministry for Development Cooperation and the STD2, Contract Nr TS2-M-0274, of the Commission of the European Communities

REFERENCES

1 VERMEULEN AN, PONNUDURAI T, BECKERS PJA, VERHAVE JP, SMITS MA & MEUWISSEN JHETH 1985 Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito *J Exp Med 162* 1460-1476

2 VERMEULEN A, DEURSEN VAN J, BRAKENHOFF RH, LENSEN THW, PONNUDURAI T & MEUWISSEN JHETH 1986 Characterisation of *Plasmodium falciparum* sexual stage antigens and their biosynthesis in synchronised gametocyte cultures *Mol Biochem Parasitol 20* 155-163

3 KUMAR N & CARTER R 1984 Biosynthesis of the target antigens of antibodies blocking transmission of *Plasmodium falciparum Mol Biochem Parasit 13* 333 342

4 VERMEULEN AN, ROEFFEN WFG, HENDERIK JBJ, PONNUDURAI T, BECKERS PJA & MEUWISSEN JHETH 1985 *Plasmodium falciparum* transmission blocking antibodies recognize monovalently expressed epitopes *Dev Biol Stand 62* 91 97

5 GRAVES PM, CARTER R, BURKOT TR, QUAKYI IA & KUMAR N 1988 Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera *Parasite Immunol 10* 209 218

6 ALTMAN DG 1991 *Practical Statistics for Medical Reseach* Londen, Chapman and Hall

7 LANDIS JR & KOCH GG 1977 The measurement of observed agreement for categorical data *Biometrics 33* 159-174

8 PONNUDURAI T, LENSEN AHW, LEEUWENBERG ADEM & MEUWISSEN JHETH 1982 Cultivation of fertile *Plasmodium falciparum gametocytes* in semi-automated systems I Static cultures *Trans R Soc Trop Med Hyg 76* 812-818

9 PONNUDURAI T, GEMERT VAN GJA, BENSINK T, LENSEN AHW & MEUWISSEN JHETH 1987 Transmission-blockade of *Plasmodium falciparum* its variability with gametocyte numbers and concentration of antibody *Trans R Soc Trop Med Hyg 81* 491-493

10 PONNUDURAI T, LENSEN AHW, GEMERT VAN GJA BENSINK MPE BOLMER M & MEUWISSEN JHETH 1989 Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes

Parasitology 98 165-173

11 WILLIAM CB 1937 The use of logarithms in the interpretation of certain entomological problems *Ann App Biol 24* 404-414

12 TCHUINKAM T, MULDER L, DECHERING K, STOFFELS H, VERHAVE JP, CARNEVALE P, MEUWISSEN JHETH & ROBERT V 1993 Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* in Cameroon Infectivity of gametocytes of naturally infected gametocyte carriers *Trop Med Parasitol 44* 271-276

13 MULDER B, GAZIN G, EGGELTE TA & COT M 1992 Increase of chloroquine resistance *in vivo* of *Plasmodium falciparum* over two years in Edea, south Cameroon *Trans R Soc Trop Med Hyg 86* 376

14 VERHAVE JP, LEEUWENBERG ADEM, PONNUDURAI T, MEUWISSEN JHETH & VAN DRUTEN JAM 1988 The biotin-streptavidin system in a two site ELISA for the detection of plasmodial sporozoite antigen in mosquitoes *Parasite Immunol 10* 17-31

15 WILSON MB, Nakane PK, 1978 Immunofluorescence and Related Staining Techniques, 215 *Elsevier, Amsterdam*

16 CARTER R, GRAVES PM, KEISTER DB & QUAKYI IA 1990 Properties of epitopes of Pfs48/45, a target of transmission-blocking monoclonal antibodies, on gametes of different isolates of *Plasmodium falciparum Parasite Immunol 12* 587-603

17 GRAVES PM, DOUBROVSKY A, SATTABONGKOT J & BATTISTUTTA D 1992 Human antibody responses to epitopes on the *Plasmodium falciparum* gametocyte antigen Pfs48/45 and their relationship to infectivity of gametocyte carriers *Am J Trop Med Hyg 46* 711-719

18 ROEFFEN W, PJA BECKERS, K TEELEN, T LENSEN, RW SAUERWEIN, JHETH MEUWISSEN & W ELING 1994 *Plasmodium falciparum* A comparison of the activity of Pfs230 specific antibodies in an assay of transmission blocking immunity and specific competition ELISA Exp Parasitol **79** in press

19 NAOTUNNE T DE S, KARUNAWEERA ND, MENDIS KN & CARTER R 1993 Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates *Immunology 78* 555 562

20 CARTER R, KUMAR N, QUAKYI IA, GOOD M, MENDIS K, GRAVES P & MILLER L 1988 Immunity to sexual stages of malaria parasites *Progess of Allergy 41* 193-214

Association between anti-Pfs48/45 reactivity and *Plasmodium falciparum* tranmisson-blocking activity in sera from Cameroon.

W. ROEFFEN[1], B. MULDER[1,2], K. TEELEN[1], M. BOLMER[1], W. ELING[1], G.A.T. TARGETT[3], P.J.A. BECKERS[1] AND R. SAUERWEIN[1]

[1] *university of nijmegen, department of medical microbiology, nijmegen, the netherlands.*
[2] *Malaria department, o.c.e.a.c./orstom, yaounde, cameroon*
[3] *london school of hygiene & tropical medicine, london, england*

Published in: *Parasite Immunology* (1996). **18**:103-109.

ABSTRACT

Pfs48/45, a sexual stage parasite protein doublet of *P falciparum*, is a target of antibodies which inhibit the development of the parasite in the mosquito Twenty eight (54%) out of 52 sera of gametocyte carriers from Cameroon reduced infectivity in the mosquito membrane feeding bioassay to less then 20% of the controls These 52 sera were analysed by competition ELISAs for the presence of antibodies capable of competing the binding of six mAbs directed against five different epitopes on Pfs48/45 The percentage of these 52 Cameroon sera that competed with one of the mAbs ranged from 13% (epitope I) to 33% (Epitope IIc) Comparison of activity in the transmission-blocking (TB) assay ($\geq$80%) and in the Pfs48/45 competition ELISA show a relative specificity of 100% (24 of 24) and a relative sensitivity of 75% (21 of 28) Non blocking sera showed no competition with any of the mAbs These mAbs were further used to study the diversity of epitopes among isolates of *P falciparum* using a two-site ELISA MAbs against epitope I, III and V reacted with four different isolates whereas epitope II could be subdivided into three epitopes None of the isolates reacted with mAb 3G12 (epitope IV) Using these four different isolates, the competition ELISA titer varies from 1/20 to 1/80 and no significant differences are found between the isolates except for epitope II where only three out of 11 positives for epitope IIa were also positive for epitiope IIc

INTRODUCTION

Gametocytes of *P falciparum* synthesize molecules of apparent M_r on SDS-PAGE of 230 kDa and 48/45 kDa (Pfs230 and Pfs48/45) which are expressed on the surface of macrogametes and zygotes [12,23] A number of monoclonal antibodies (mAbs) against the Pfs48/45 protein doublet are able to block transmission of the parasites to the mosquito vector [4,17,20,23]

Three epitopes of Pfs48/45 were identified by Carter *et al* [2,3] MAb to epitope I (mAb 3E12) blocks transmission effectively but mAbs to epitopes II (mAb IA3 B8) and III (mAb IIC5 B10) were ineffective on their own but able to reduce transmission when used in combination Another epitope (IV) was defined by its reactivity with a non blocking mAb 3G12, that bound Pfs48/45 only under reduced conditions on a Western immunoblot [4] Diversity in epitope II was found in two Liberian isolates when gametocytes of 15 different isolates from a variety of endemic areas were tested for binding of mAbs to Pfs48/45 by indirect immunofluorescence and immunoprecipitation [6] Epitope II is subdivided into epitopes a, b and c of which a and c represent the genetic variants [4] Similar findings were obtained by Foo *et al* [5] in a series of 33 *P falciparum* isolates from Malaysia, variation exists in epitope IIa and IIc, while epitope I, IIb, III and IV are

markedly conserved Using the PCR technique nucleotide and amino acid sequence differences were observed between the Pfs48/45 genes of 8 different strains of *P falciparum* by Kocken *et al* [11] The results showed that the amino acid residue at position 254 in Pfs48/45 determined whether this antigen will bind to a mAb specific for epitope IIa or epitope IIc

The availability of a larger panel of mAbs permitted the identification of five independent antibody binding sites on Pfs48/45 [20] and TB-activity appeared no longer to be restricted to epitope I recognition i e mAbs against epitope II, III and V were all able to block transmission Furthermore, blocking was not associated with particular epitopes and blocking and non-blocking mAbs competed for the same binding site

Anti-Pfs48/45 antibodies are widely present in sera from individuals living in malaria endemic areas [5,6,18] Since Pfs48/45 is a target protein for TB antibodies, a study was carried out to analyse the possible association between anti Pfs48/45 antibodies and the capacity to reduce transmission in the membrane feeding bioassay in sera from naturally infected individuals This classical test is a laborious and time consuming assay that cannot easily be transferred to endemic areas and used for epidemiological studies Therefor, an easy field test is required to study the possible impact of TB-immunity for malaria The presence of anti Pfs48/45 antibodies in sera was previously determined by a competi tion ELISA using an anti Pfs48/45 mAb recognizing epitope I (mAb 32F3, [18] In that study TB activity was predicted with a specificity of 96% and a sensitivity of 44% in sera of gametocyte carriers from Cameroon Here we further extend these findings by using a panel of 6 different mAbs directed against epitope I, II, III and V In addition, the specificity of epitope reactivity was studied in 4 isolates

MATERIALS AND METHODS

Parasites

Mature gametocytes of *P falciparum* (isolate NF54 (Amsterdam, airport strain), 7G8 (Brazil, Burkot 1984), PNG94 (Papua New Guinea, Graves 1988a), TT1 (Cameroon strain)) were produced in an automated static culture system as described by Ponnudurai *et al* [14] Gametocytes were isolated as previously described [19] The purified gametocytes were stored at -70°C until used

Monoclonal antibodies and sera

MAbs 32F1 and 32F3 have been described by Vermeulen *et al* [23] MAbs 3E12 (epitope I), IA3 B8 (epitope IIa), IIC5 B10 (epitope III) and 3G12 (epitope IV) were a kind gift of Dr R Carter MAbs 82D6 A10 (epitope IIc) and 84A2 A4 (epitope V) have been

described by Targett *et al* [20]

Field sera 52 sera were collected from gametocyte carriers (aged between 6-36 years) recruited at a dispensary in Yaounde, Cameroon [22]

Sp3 and Sp5 are sera collected from a *P falciparum* infected Dutch expatriate respectively 3 and 5 weeks after return from Cameroon who worked there for more than 30 years The sera Sp3 and Sp5 were collected while the patient was a *P falciparum* gametocyte carrier

Serum (St) is from a Dutch expatriate who worked in an endemic malaria area in Tanzania for more than 30 years

Negative control (N56) A pooled serum sample obtained from Dutch bloodbank donor volunteers, without any history of malaria, collected in the absence of a potent infection

Transmission-blocking assay

A modification of the bioassay, using membrane feeders to feed *A gambiae* mosquitoes, was used to determine TB-capacity of serum samples [15,16,23] Percentage reduction of oocysts was calculated according to the equation $R=(T_c - T_t) / T_c \times 100\%$ where T_c is the geometric mean of oocyst number observed after feeding with three control sera and T_t is the geometric mean of oocyst number observed after feeding with the test serum [19]

Enzyme-linked immunosorbent assays (ELISAs)

Pfs48/45 of different isolates was extracted in 25 mM TNP (Tris HCl (pH 8 0), 150 mM NaCl, 0 5% Nonidet P40), 1 mM phenylmethylsulphonyl fluoride and 1 μg/ml each of DNase and RNase Insoluble debris was pelleted by centrifugation (16,000g, five minutes at room temperature) and the supernatant was stored at -20^{0}C until use

Two-site ELISAs and two-site competition ELISAs using gametocytes of different isolates and horseradisch peroxidase (HRPO) labelled Protein-A purified mAbs were carried out as previously described [18,19] Briefly, wells of microtiterplates were coated with 50 μl of anti-Pfs48/45 mAb (10 μg/ml) in PBS for 30 minutes Wells were saturated with 150 μl of 5% foetal calf serum (FCS) in PBS for 30 min After three washes with PBS, the wells were incubated for two hrs with 50 μl of the NP40 gametocyte extract, containing the equivalent of 150,000 gametocytes diluted in TNP Following three washes with PBS, wells were incubated with 50 μl HRPO-labelled anti-Pfs48/45 mAb (2 μg/ml) for two hours After four washes with PBS, wells were incubated with 60 μl TMB (0 25 mM 3,3',5,5'-tetra-methyl-benzidine, 0 7 mM H_2O_2, 0 1 M sodiumacetate pH 5 5)

substrate solution for 20 minutes The enzyme reaction was stopped by adding 60 μl 4 N H_2SO_4 and the optical density (OD) was determined on an ELISA-reader at 450 nm (Titertek Multiskan MCC/340) All incubations were carried out at room temperature.

For the competition ELISA, Pfs48/45 was captured from a TNP extract in microtiterplates as described above. After three washes with PBS, wells were incubated with a mixture of 30 μl test sample and 30 μl HRPO-labelled anti-Pfs48/45 mAb (1 μg/ml) for 2 hours Competition ELISAs were carried out using serial two-fold dilutions of unlabelled anti-Pfs48/45 mAbs (ranging from 0.1 - 10 μg/ml) or human sera diluted in PBS containing 0.1% FCS ranging from 1/10 to 1/2560 dilution in competition with one of the HRPO-labelled mAbs The plates were washed, incubated with substrate and the absorbance read at 450 nm as above

For purposes of standardization each ELISA was done with a positive and a negative control The competition titer was defined as the dilution of the test serum that resulted in the same OD value as the mean +2SD of OD values for 14 non-exposed control sera Sera with a dilution titer $\geq 1/20$ are considered positive [18].

RESULTS

Immunoreactivity of anti-Pfs48/45 mAbs using two-site ELISAs

Epitope specificity of the mAbs was determined by competition between labelled and unlabelled mAbs in two-site competition ELISAs and determining the binding characteristics to *P falciparum* isolates which are known to be different in Pfs48/45 phenotype. Checkerboard titrations with labelled mAbs and antigen extracts were performed to establish optimal conditions for Pfs48/45 detection None of the labelled mAbs bound to Pfs48/45 when captured by the corresponding unlabelled mAbs indicating that all epitopes are non-repetitive

Table 1 shows the reactivity of 3 anti-Pfs48/45 mAbs recognizing epitope II with 4 different geographical isolates. While mAb 1A3 B8 reacts exclusively with isolate 7G8, mAb 82D6 A10 reacts with all isolates except 7G8. These data confirm previous findings by Graves *et al* 1985 and Foo *et al.* 1991 showing variation in epitope II between different isolates as detected by immunoprecipitation or immunofluorescence microscopy HRPO-labelled mAb 32F3 (epitope I), IIC5.B10 (epitope III) or 84A2.A4 (epitope V) reacted with these 4 different isolates No reactions were found in our two-site ELISAs using the four isolates as antigen with mAb 3G12 or the HRPO-labelled mAb 3G12 whereas this mAb reacted positive in the immuno-fluorescence assay using air dried, aceton fixed gametocytes of isolate NF54 or 7G8 (data not shown)

TABLE 1 Two-site ELISA with HRPO labelled anti-Pfs48/45 mAbs of the different epitopes using antigen from 4 different isolates captured by a mAb

epitope	second	Isolate[$]			
	HRPO-mAb	NF54	PNG	7G8	TT1
I	32F3	+	+	+	+
II a	IA3 B8		-	+	-
II b	32F1	+	+	+	+
II c	82D6 A10	+	+	-	+
III	IIC5 B10	+	+	+	+
IV	3G12		-	-	-
V	82D6 A10	+	+	+	+

[$] Isolate NF54 (Amsterdam, airport strain) 7G8 (Brazil), PNG (Papua New Guinea) and TT1 (Cameroon), (+) positive reaction, ( ) no reaction

TB-activity and Pfs48/45 competition titer of human sera

The reactivity of mAbs with different isolates including NF54 and a Cameroonian isolate as described in table 1 shows comparable reactivity between NF54 and TT1 from Cameroon A panel of 52 sera from gametocyte carriers in Yaounde was used to study for TB-capacity and presence of natural anti-Pfs48/45 antibody reactivity using NF54 as isolate (Table 2) Twenty-eight (54%) out of 52 sera of gametocyte carriers from Cameroon reduced transmission (mean oocyst number) in the bioassay to less than 20% of the value observed in malaria negative control sera and 6 of these sera completely blocked transmission The proportion of sera competing for a given epitope was 13% (7 sera) for epitope I, 21% (11 sera) for epitope IIb, 29% (15 sera) for epitope III, 31% (16 sera) for epitope V and 33% (17 sera) for epitope IIc Twenty-one (40%) of the 52 sera were able to compete binding to any epitope and were always able to reduce transmission more than 80% Out of the 28 transmission-reducing sera, 7 (25%) were positive for epitope I, 11 (39%) for epitope IIb, 15 (54%) for epitope III, 16 (57%) for epitope V and 17 (61%) for epitope IIc The combined Pfs48/45 competition ELISAs were positive in 21 (75%) of the 28 sera with a transmission-reducing activity of more than 80% None of the non-blocking sera (24 of 52) exhibited activity in any of the anti Pfs48/45 competition ELISAs Seven

TABLE 2 Reactity of 52 sera from *P falciparum* gametocyte carriers in the bioassay for transmission-reducing activity and the competition test for Pfs48/45 epitopes using NF54 isolate

		Epitope of Pfs48/45[&]				
Serum	**R[#]**	**I**	**IIb**	**IIc**	**III**	**V**
1	98	160*	20	40	20	40
2	100	40	80	80	80	40
3	96	40	160	80	40	80
4	100	640	40	80	80	40
5	89	160	-	40	40	20
6	91	80	-	40	40	40
7	88	40	-	-	-	-
8	91	-	160	40	40	80
9	97	-	160	40	20	40
10	84	-	20	40	40	40
11	82	-	80	40	40	40
12	97	-	40	20	-	20
13	100	-	20	20	-	-
14	90	-	20	-	-	-
15	90	-	-	40	40	40
16	81	-	-	40	40	40
17	80	-	-	20	40	20
18	100	-	-	20	20	-
19	81	-	-	20	-	20
20	94	-	-	-	20	-
21	83	-	-	-	-	20
22	96	-	-	-	-	-
23	99	-	-	-	-	-
24	100	-	-	-	-	-
25	90	-	-	-	-	-
26	100	-	-	-	-	-
27	99	-	-	-	-	-
28	90	-	-	-	-	-
29-52	<80	-	-	-	-	-

[&] – Competing antibodies for epitope I, IIb, IIc, III and V were respectively mAb 32F3, 32F1, 82D6 A10, IIC5 B10 and 84A2 A4, [#] = percentage reduction in TB-assay, * = Reciprocal serum titer, (-) = Titer <20

out of 28 TB-sera did not compete the mAbs of the different epitopes used in this study.

The negative control serum does not reduce the OD reading of the labelled mAbs for any of the Pfs48/45 epitopes. The activity of the TB-assay (positive if $\geq 80\%$, negative if $< 80\%$) was compared to the results of the competition ELISA for Pfs48/45 (positive if titer ≥ 20, negative if < 20) in a two by two table. The results of these data showed a relative specificity i.e. proportion negative in both tests of 100% (24 out of 24) and a relative sensitivity, i.e. proportion blockers positive in the competition ELISA of 75% (21 out of 28).

TABLE 3. Reciprocal competition titer for different Pfs48/45 epitopes in human sera with anti-Pfs48/45 mAbs.

	Epitope of Pfs48/45#					
Serum	**I**	**IIa**	**IIb**	**IIc**	**III**	**V**
St2	320*	320	320	320	320	320
Sp3	640	640	-	80	2560	80
Sp5	2560	1280	-	80	2560	80
N56	-	-	-	-	-	-

St2 = Positive control serum; Sp = Serum samples from a Dutch expatriate collected in week 3 (Sp3) and 5 (Sp5) after returning from Cameroon; N56=A pool from Dutch blood donors with no history of malaria; * = Reciprocal serum titer; (-) = Titer < 20; # = For all competition test NF54 isolate was used execpt for epitope IIa where 7G8 isolate was used as source of Pfs48/45. Competing antibodies for epitope I, IIa, IIb, IIc, III and V were respectively mAb 32F3, IA3.B8, 32F1, 82D6.A10, IIC5.B10 and 84A2 A4.

Antibody reactivity with Pfs48/45 epitopes of different isolates.

Table 3 shows the results of two-site competition ELISAs with serial dilutions of malaria negative serum (N56) and sera from 2 Dutch expatriates (St2, Sp3 and Sp5). Sera from these Dutch expatriates completely block transmission (data not shown). Serum St2 competes with all HRPO-labelled mAbs for all epitopes in a dilution of 1/320. Sera from donor Sp (Sp3, Sp5), with a gametocyte positive thick smear 3 and 5 weeks after returning from Cameroon, competes with all epitopes except epitope IIb. Reactivity of the Sp samples against epitopes IIc and V was much lower than reactivity to epitopes I, IIa and III. In addition, reactivity against Pfs48/45 was higher in the expatriate sera, relative to

the sera of the gametocyte carriers (Table 2) Sp3, Sp5 (Table 3), and also ten out of 21 sera from gametocyte carriers, positive for at least one of the Pfs48/45 epitopes (Table 2), did not show any competition with mAb 32F1 recognizing epitope IIb Only for epitope I we see an increase in titer for donor Sp In contrast to the mAb reactivity (Table 1) a positive reaction was found for both epitopes IIa and IIc with sera from these 2 Dutch expatriates

TABLE 4 Pfs48/45 reactivity against different isolates in sera from *P falciparum* gametocyte carriers using the epitope specific competition ELISAs

	Isolate			
Pfs48/45 reactivity (n=20)	**NF54**	**TT1**	**PNG94**	**7G8**
positive with at least one epitope	12	9	12	10[c]
epitope II[#]	11[a]	8[a]	10[a]	3[b]
epitope III[#]	9	7	9	8[d]
epitope V[#]	11	9	10	8

[#] = epitope IIa as defined by mAb IA3 B8, IIc by mAb 82D6 A10, epitope III by mAb IIC5 B10, epitope V by mAb 84A2 A4, [a] = positive for epitope IIc, [b] – positive for epitope IIa, [c] = 19 sera tested, [d] = 18 sera tested

With a selection of 20 out of 52 sera from gametocyte carriers competition ELISAs against different epitopes of Pfs48/45 were carried out using four *P falciparum* isolates The results are described in Table 4 Eight of the 20 sera were negative in the competition ELISAs with NF54 as well as with isolates TT1, PNG94 and 7G8 The 12 competition positive with NF54 were also tested with the 3 other isolates All sera recognized the PNG isolate, but three out of 12 sera did not compete Pfs48/45 mAbs binding to TT1, one out of 11 did not show positive competition with 7G8 Serum titers varied between 1/20 and 1/80 showing no major difference between the isolates In 3 sera (4, 8 and 15 in Table 2) we find competition for both epitope IIc and IIa

DISCUSSION

Antibody responses to gametocyte antigens occur in individuals exposed to *P falciparum* [7,8,9,10,18,19,21] including those who had experienced only primary infections [13] Competition ELISAs using six mAbs, recognizing different epitopes of Pfs48/45 were

carried out to determine the binding specificities of the naturally acquired antibodies to Pfs48/45 Our results confirm the reactivity of different mAbs with the epitopes described by Carter *et al* [2,4] and Targett *et al* [20] No reaction was found in our two-site ELISAs with mAb 3G12. Enhancement of binding of HRPO-labelled mAbs in the presence of sera to the captured antigen was never seen in these competition ELISAs (data not shown) in contrast to findings by Targett *et al.* [21] or Graves *et al* [8,10]

In a panel of sera from gametocyte carriers, 28 sera exhibited more than 80% transmission-reducing activity. Twenty-one of these gametocyte carriers were positive in anyone of a Pfs48/45 competition ELISA but individual sera might have only one specificity or any combination of two or more specificities. It is interesting to note that considerable variation was seen in the antibody responses to each of the five epitopes on the Pfs48/45 molecule, all of which are associated with transmission-reducing activity The combined Pfs48/45 competition ELISAs predict 75% (21 out of 28 sera) of the observed transmission-reducing activity that is higher than 80% No positive Pfs48/45 competition reaction was observed in sera that reduced transmission in the bioassay less than 80%.

A similar competition ELISA was used by Graves *et al.* [8,9,10] and Ong *et al* [13] with mAbs including 3G12 against 3 different epitopes and X10 or XP12 as source of Pfs48/45 Graves *et al.* [10] showed a significant association between lack of infectivity of *P falciparum* gametocyte carriers and recognition of epitope IIa on Pfs48/45 by antibodies in their sera In another study, mAb IIC5 B10 was used to compete with a panel of Papua New Guinea sera The results of the competition ELISA did not correlate with TB-activity observed in these field sera [7,8] We have previously described a correlation between suppression of infectivity of *in vitro* cultured gametocytes to mosquitoes in a bioassay and reactivity in an anti-Pfs48/45 two-site competition ELISA by immune sera [18]. In that study Pfs48/45 was captured from a NF54 parasite extract by mAb 32F1 (epitope 2b) and the sera competed with mAb 32F3 (epitope 1). The results showed that the competition ELISA for one epitope had a relative sensitivity of 44% and a relative specificity of 94% The data described in this paper exhibit a 100% specificity and an overall relative sensitivity of 75%. It remains to be determined why 25% of the sera with TB-activity are negative in any of the competition ELISAs In addition, three sera bound to NF54 derived Pfs48/45 did not recognize TT1 parasites, whereas another serum did not recognize 7G8 parasites Possible explanations for these observations are. a) insufficient sensitivity of the competition ELISA. Competition titers are relatively low (1/20) in cases where epitopes of NF54 but none of the other strains are recognized, b) TB-activity depends on antibody reactivity against as yet unknown Pfs48/45 epitope(s), c) antibody reactivity against other molecules, d) antibody-independent reactions

Studies on different isolates of *P falciparum* show that epitopes I, IIb and III are conserved while variations exist in expression of epitope IIa and IIc (Table 4). This result confirms earlier observations from a larger study by Foo *et al* [5] Appropriate isolates (NF54 for IIc and 7G8 for IIa) were used in the competition-ELISAs to determine presence of IIa and/or IIc reactivity in field sera. Competition reactivity for both epitope IIa and IIc was found in sera from the two Dutch expatriates and in 3 sera of Cameroon Foo *et al* [5] found in one isolate in which individual gametocytes expressed both forms (IIa and IIc) using the immunofluorescence technique It implies a more complex explanation of the relationship between these subepitopes than allelic alternatives These findings of expressing both forms has to be proven with different isolates by the PCR technique of Kocken *et al* [11]

The proportion of sera from 52 gametocyte carriers with antibodies competing individual Pfs48/45 mAb ranged from 13% to 33% suggesting epitope restricted immune reactivity The TB-bioassay is a labor-intensive and time-consuming test. As shown in this study the majority of TB-sera from Cameroon can be predicted by Pfs48/45 competition ELISA which may be a useful tool in further epidemiological studies on TB-immunity.

ACKNOWLEDGEMENTS

We are grateful to Marianne Sieben for production and purification of monoclonal antibodies, to Arianne Huisman and Geertjan van Gemert for parasite cultures and transmission experiments, to Timoleon Tchuinkam and Julienne Essong for detection of gametocyte carriers and collection of sera and to JHETh Meuwissen for critically reading and discussion of the manuscript The generous gift of various mAbs by Dr R Carter, Division of Biological Sciences, Edinburgh, England is thankfully acknowledged.

This research was supported by the Dutch Ministry for Development Co-operation (DGIS/-SO) contract nr NL002701 This investigation also received financiel support from CEC contract nr TS3-C193-0229 and The Wellcome Trust

REFERENCES

1 BURKOT TR, WILLIAMS JL & SCHNEIDER I (1984) Infectivity to mosquitoes of *P falciparum* clones grown in vitro from the same isolate *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 335

2 CARTER R, BUSHELL G, SAUL A, GRAVES PM & KIDSON C (1985) Two apparently nonrepeated epitopes on gametes of *P falciparum* are targets of transmission blocking antibodies *Infection and Immunity* **50**, 102-106

3 CARTER R, KUMAR N, QUAKYI IA, GOOD M, MENDIS K, GRAVES P & MILLER L (1988) Immunity to sexual stages of malaria parasites *Progess of Allergy* **41**, 193-214

4 CARTER R, GRAVES PM, KEISTER DB & QUAKYI IA (1990) Properties of epitopes of Pfs48/45, a target of transmission-blocking monoclonal antibodies, on gametes of different isolates of *P falciparum* *Parasite Immunol* **12**, 587-603

5 FOO A, CARTER R, LAMBROS C, GRAVES P, QUAKYI I, TARGETT G, PONNUDURAI T & LEWIS G (1991) Conserved and variant epitopes of target antigens of transmission-blocking antibodies among isolates of *P falciparum* from Malasia *American Journal of the Tropical Medicine and Hygiene* **44**, 623-631

6 GRAVES PM, CARTER R, BURKOT TR, RENER J, KAUSHAL DC & WILLIAMS JL (1985) Effect of transmission-blocking antibodies on different isolates of *P falciparum* *Infection and Immunity* **48**, 611-616

7 GRAVES PM, CARTER R, BURKOT TR, QUAKYI IA & KUMAR N (1988a) Antibodies to *P falciparum* gamete surface antigens in Papua New Guinea sera *Parasite Immunology* **10**, 209-218

8 GRAVES PM, WIRTZ RA, CARTER R, BURKOT TR, LOOKER M & TARGETT GAT (1988b) Naturally occuring antibodies to an epitope on *P falciparum* gametes detected by monoclonal antibody-based competitive Enzyme-linked Immunosorbend Assay *Infection and Immunity*, **56**, 2818-2821

9 GRAVES PM, DOUBROVSKY A, CARTER N & BECKERS P (1990) High frequency of antibody response to *P falciparum* gametocyte antigen during acute malaria infections in Papua New Guinea highlanders *American Journal of Tropical Medicine and Hygiene* **44**, 515-520

10 GRAVES PM, DOUBROVSKY A, SATTABONGKOT J & BATTISTUTTA D (1992) Human antibody responses to epitopes on the *P falciparum* gametocyte antigen Pfs48/45 and their relationship to infectivity of gametocyte carriers *American Journal of Tropical Medicine and Hygiene* **46**, 711-719

11 KOCKEN CHM, MILEK RLB, LENSEN THW, KASLOW DC, SCHOENMAKERS JGG & KONINGS RNH (1995) Minimal variation in the transmission-blocking vaccine candidate Pfs48/45 of the human malaria parasite *P falciparum* *Molecular and Biochemical Parasitology* **69**, 115-118

12 KUMAR N & CARTER R (1984) Biosynthesis of the target antigens of antibodies blocking transmission of *P falciparum* *Molecular and Biochemical Parasitology* **13**, 333-342

13 ONG CSL, ZHANG KY, EIDA SJ, GRAVES PM, DOW C, LOOKER M, ROGERS NC, CHIODINI PL & TARGETT GAT (1990) The primary antibody response of malaria patients to *P falciparum* sexual stage antigens which are potential transmission blocking vaccine candidates *Parasite Immunology* **12**, 447-456

14 PONNUDURAI T, LENSEN AHW, LEEUWENBERG ADEM & MEUWISSEN JHETH (1982) Cultivation of fertile *P falciparum* gametocytes in semi-automated systems I Static cultures *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76**, 812 818

15 PONNUDURAI T, VAN GEMERT GJA, BENSINK T, LENSEN AHW & MEUWISSEN JHETH (1987) Transmission blockade of *P falciparum* its variability with gametocyte numbers and concentration of antibody *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 491-493

16 PONNUDURAI T, LENSEN AHW, VAN GEMERT GJA, BENSINK MPE, BOLMER M & MEUWISSEN JHETH (1989) Infectivity of cultured *P falciparum* gametocytes to mosquitoes *Parasitology* **98**, 165-173

17 RENER J, GRAVES PM, CARTER R, WILLIAMS JL & BURKOT TA (1983) Target antigens of transmission-blocking immunity on gametes of *P falciparum J of Experimental Medicine* **158,** 976-981

18 ROEFFEN W, LENSEN T, MULDER B, TEELEN K, SAUERWEIN R, VAN DRUTEN J, ELING W, MEUWISSEN JHETH & BECKERS PJA (1995a) A comparison of transmission-blocking activity with reactivity in a *P falciparum* 48/45-kD molecule-specific competition ELISA *American Journal of the Tropical Medicine and Hygiene* **52**, 60 -65

19 ROEFFEN W, BECKERS PJA, TEELEN K, LENSEN T, SAUERWEIN RW, MEUWISSEN JHETH & ELING W (1995b) *P falciparum* A comparison of the activity of Pfs230 specific antibodies in an assay of transmission-blocking immunity and specific competition ELISA *Exp Parasitology* **80**,15-26

20 TARGETT GAT, HARTE PG, EIDA S, ROGERS NC & ONG CSL (1990a) *P falciparum* sexual stage antigens immunogenecity and cell-mediated resposes *Immunological letters* **25**, 77 - 82

21 TARGETT GAT (1990b) Immunity to sexual stages of human malaria parasites Immune modulation during natural infections, antigenic determinants, and the induction of transmission-blocking immunity *Scandinavian Journal of Infection Diseases* Supplement **76**, 79 88

22 TCHUINKAM T, MULDER L, DECHERING K, STOFFELS H, VERHAVE JP, CARNEVALE P, MEUWISSEN JHETH & ROBERT V (1993) Experimental infections of *A gambiae* with *P falciparum* in Cameroon Infectivity of gametocytes of naturally infected gametocyte carriers in Cameroon factors influencing the infectivity of mosquitoes *Tropical Medicine and Parasitology* **44**, 271-276

23 VERMEULEN AN, PONNUDURAI T, BECKERS PJA, VERHAVE JP, SMITS MA & MEUWISSEN JHETh (1985) Sequential expression of antigens on sexual stages of *P falciparum* accessible to transmission blocking antibodies in the mosquito *Journal of Experimental Medicine* **162,** 1460 1476

CHAPTER 7

Longitudinal changes in antibodies and transmission-blocking activity of human sera after a *Plasmodium falciparum* malaria infection.

W. ROEFFEN, K. TEELEN, M. BOLMER, W. ELING AND R. SAUERWEIN.

University of Nijmegen; Department of Medical Microbiology, section Parasitology

ABSTRACT

A longitudinal study was carried out with blood samples from five adult *P.falciparum* malaria patients. Sera were tested for their transmission-blocking capacity and antibodies against antigens of *P.falciparum* asexual and sexual parasites detected by a) immunofluorescence assay (IFA) and b) competitive ELISAs against different epitopes of Pfs48/45 and Pfs230. Transmission-blocking (TB) activity was present in sera from patient Ha, Sp and Ro for at least 17, 83 and 12 weeks respectively. A close association was found between increase and decrease in antibody titres to epitope III of Pfs48/45 in the competition ELISA and the TB-activity of the individual sera. The prevalence of antibodies against asexual or sexual parasites was significantly higher in the three patients with a malaria history, than in two patients with a first infection and no gametocytes.

INTRODUCTION

Plasmodium species are transmitted to the *Anopheles* vector by ingestion of gametocytaemic blood. Gametocytes of *P.falciparum* synthesize Pfs230 and Pfs48/45 which are expressed on the surface of macrogametes and zygotes [9,17,26]. At least five epitopes of Pfs48/45 or Pfs230 were identified respectively by Carter *et al.* [1,2], Targett *et al.* [23] and by Read *et al.* [16] and confirmed by Roeffen *et al.* [18,19]. TB-activity was not considered to be associated with particular epitopes as blocking- and non-blocking mAbs competed for the same binding site in competition-ELISAs. A number of studies indicate that antibody responses to epitopes of Pfs48/45 and Pfs230 are part of the response observed after several malaria episodes [3,5,7] and that some epitopes are conserved in many isolates [4]. Immuno-precipitation with sera from people living in endemic areas precipitated radio-iodinated Pfs48/45 and Pfs230. A good correlation was found between TB-activity of the serum and antibody response against Pfs230 using the immunoprecipitation assay in sera from Papua New Guinea. In addition, no such correlation was found for the Pfs48/45 antibody reactivity [5. On the other hand, in a study using sera from Sri Lanka no correlation was found between TB-activity and the presence of antibodies against Pfs230 using the same immunoprecipitation technique [15]. We have previously described an association between TB-activity of *in vitro* cultured gametocytes to mosquitoes in a bioassay and reactivity in an anti-Pfs48/45 two-site competition ELISA in several sets of sera [18,20]. The results show a 100% specificity and an overall relative sensitivity of 75% for prediction of TB-activity using 6 anti-Pfs48/45 mAbs recognizing different epitopes. No correlation was found between TB-activity and reactivity in an anti-Pfs230 two-site competition ELISA in sera from gametocyte carriers [19].

The aim of the present study was to determine the prevalence, development and

persistence of naturally occuring antibodies against Pfs230 and Pfs48/45 and their association with TB-activity. Five patients admitted to hospital with a *P.falciparum* infection were studied for a period of up to 80 weeks. Parasitological, serological and *in vitro* transmission data were collected to study conditions for development and persistence of asexual and sexual stage antibodies and their relation to TB-immunity. In addition, this study describes development, persistence and fading of TB-immunity after radical cure of a *P.falciparum* infection.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 5 patients (Ha, Sp, Ro, Ja and Gr) with clinical malaria at presentation at the Academical Hospital St. Radboud in Nijmegen. Patient Ha, Sp, Ja and Gr were all Europeans. Patient Ha and Sp were expatriates living in malaria endemic areas in East-Africa (Mozambique, Kenia) and West-Africa (Cameroon) for respectively 12 and 30 years with regular attacks of clinical malaria. Two patients (Ja and Gr) presented with a first malaria infection after returning from Cameroon. Patient Ro was borned in Irian Jaya and has lived there for 25 years thereby experiencing several malaria infections. Thereafter she lived in the Netherlands for 7 years. After a short visit to Irian Jaya she returned with a malaria infection.

Patient Ja and Gr indicated that they visited the hospital within a week after the first clinical symptoms, whereas patient Ha, Sp and Ro complaints were present for two weeks or more. Blood samples were obtained at different time points after treatment (see Table 1) for a) parasitologic examination and b) serologic analysis (stored at -20^{0}C until use).

Pooled serum obtained from Dutch bloodbank donors, without a history of malaria, was used as a negative control (N56).

Parasitological examinations.

P. falciparum malaria was diagnosed in Giemsa-stained thick and thin blood films. Asexual parasites and gametocytes were counted per 500 leukocytes and the total number of parasites was calculated against the number of leukocytes per μl. A blood film was considered negative when no *P.falciparum* parasite was found per 2000 leukocytes.

Monoclonal antibodies (mAbs)

Anti-Pfs230 mAbs 18F25, 63F6D7 and 63F3C8 have been described previously [19]. Anti-Pfs48/45 mAbs IA3.B8 (epitope IIa) and IIC5.B10 (epitope III) were a kind gift of

Dr R Carter MAbs 82D6 -A10 (epitope IIc) and 84A2 A4 (epitope V) were a kind gift of Dr G Targett MAbs 32F3 (epitope I) and 32F1 (epitope IIb) have been described previously [18,26]

Parasites

Mature gametocytes of *P falciparum* (isolate NF54, Amsterdam airport strain, and 7G8, Brazilian strain) were produced in an automated static culture system as described by Ponnudurai *et al* [12] Mature gametocytes were a) isolated as previously described [19] and stored at -70°C until used or b) allowed to undergo gametogenesis for 30 min by resuspension at a 10% haematocrit in Foetal Calf Serum The gametes were isolated at 4°C as previously des-cribed [26] and used directly for suspension-IFA

Gametocytes of isolate NF54 or 7G8 were extracted in 25 mM Tris-HCl (pH 8 0) supplemented with 150 mM NaCl, 0 5% Nonidet P40 (NP40), 1 mM phenylmethylsulphonyl fluoride and 1 μg/ml each of DNase and RNase Insoluble debris was pelleted by centrifugation (16,000g, 5 minutes at room temperature) and the supernatant was stored at -20°C until used as source of Pfs48/45 or Pfs230 in the capture ELISAs

Transmission blocking assay

In a previously described bioassay [13,14,26], *A gambiae* mosquitoes were allowed to feed on membrane feeders with cultured NF54 gametocytes in the presence of test or control sera, heat inactivated at 56°C for 30 minutes, and 12 5% fresh human serum as a source of complement The percentual reduction of oocysts was calculated as described previously [19] The TB-titre is the maximum dilution of the serum still having ≥85% reduction of the oocyst number as compared to controls

Serologic analysis

a) Indirect immunofluorescence (IFA)

A mix of cultured asexual and sexual stage parasites from an isolate of *P falciparum* (NF54) were air-dried on multispot slides and incubated with two-fold dilutions of the test sera (starting with 1 20) in phosphate buffered saline (PBS) for 30 minutes The slides were rinsed with PBS and incubated with fluorochrome-conjugated goat-anti-human immunoglobuline Ig(G,M,A) for 30 minutes The slides were rinsed, washed, mounted with a mixture of 90% glycerol and 10% Tris-HCl (pH=9 0) under a coverslip, and examined under ultraviolet illumination with a Leitz microscope The end point was taken as the last dilution of the serum showing clear asexual and/or sexual stage parasites specific green fluorescence

b) Suspension-IFA (SIFA)

Surface immunofluorescence was done on suspensions of live female gametes diluted to 10^8 gametes/ml in PBS containing 10 μl packed normal human erythrocytes. 10^6 gametes were incubated with 20 μl serum dilutions for 25 min. Gametes were washed twice with PBS and incubated for a further 20 min with 50 μl of 1:125 dilution in PBS with 0.05% Evans blue of a fluorescein conjugated anti-human Ig(G,A,M). Gametes were then washed twice with PBS and resuspended in 50 μl of PBS and observed under UV illumination at a magnification of x400. The highest dilution of a serum that gave a green fluorescence of the membrane of live gametes was considered as the antibody titre. All incubations were done on ice and fluorescein conjugated mAb 32F3 was used as a control on round forms.

c) Two-site competition Enzyme-Linked Immunosorbent Assays (ELISAs)

Sera were screened for antibodies to different epitopes of Pfs48/45 and Pfs230 using two-site competition-ELISAs as described previously [18,19]. Briefly, unlabelled mAbs (10 μg/ml) were coated to plates and Pfs230 or Pfs48/45 was captured from an NP40 extract of gametocytes (see above). Horseradish peroxidase (HRPO) labelled mAbs were used as second antibody and for competition with test sera. NP40 extracts of NF54 were used as a source of Pfs230 and Pfs48/45 for all competition tests except for the analysis of Pfs48/45 epitope IIa where the 7G8 stabilate was used. Labelled anti-Pfs230 mAbs 18F25, 63F6D7 and 63F3C8 were used to detect antibodies in test sera that could competively bind to respectively epitope I, II and III whereas for Pfs48/45 labelled mAbs 32F3, IA3.B8, 32F1, 82D6.A10, IIC5.B10 and 84A2.A4 were used for the competition with antibodies that recognize respectively epitope I, IIa, IIb, IIc, III and V. The optimal concentrations of the capture antigen and the HRPO-labelled mAbs for the competition ELISAs were determined in previous studies [18,19]. The negative controls did not reduce the OD reading of the labelled mAbs for any of the Pfs230 or Pfs48/45 epitopes. The competition titre was defined as the dilution of the test serum that resulted in the same OD value as the mean +2SD OD value for 14 non-exposed control sera. Sera with a competition titre $\geq$ 1/20 are considered positive [18].

For the purpose of standardization in each ELISA a positive and a negative control were included. The optical density was read at 450 nm in a Titretek (Flow Laboratories) multiscan spectrophotometer.

RESULTS

Parasitological findings in patients.

Two patients (Ja and Gr), with no malaria history, showed a positive thick smear for

asexual *P falciparum* parasites with a parasite density of 9% and 1% respectively Gametocytes could not be found in the initial and weekly follow-up thick smears over the next 3 weeks. Patients (Ha, Sp and Ro) with clinical symptoms for malaria of at least 2 weaks, were positive for *P.falciparum*, in all three patients trophozoite density was <0.01% and the gametocyte density 0.2%, <0.01% and <0.01% (12960, 35 and 264 gametocytes/μl) respectively.

Two weeks before admission a blood sample of patient Sp was taken which was negative for malaria parasites Four days befor admission the patient treated himself with chloroquine. On admission, blood samples were collected and the thick smear was positive for *P.falciparum* gametocytes (35/μl) and no trophozoites were found. No further specific treatment was given Patient Sp showed a recrudescence with a parasitemia of <0 01% (77 trophozoites/μl and 6 gametocytes/μl) 3 weeks after admission but one week after treatment with Fansidar and Quinine the patient had cleared all parasites and remained negative thereafter. The three gametocyte carriers cleared their gametocytes within three weeks after anti-malaria chemotherapy and remained negative thereafter.

Blood samples collected at admission of patient Ha and Ro were fed a) directly and b) after replacement of plasma by normal human serum to mosquitoes The material of both patients transmitted the infection with a high oocyst load (geometric mean oocyst number of 175 and 25 respectiveley) and 90 - 100% mosquito infection rate independent of the presence of own plasma or replacement by serum Comparable observations were made one week after the start of therapy

Transmission-blocking activity

Longitudinally collected serum samples of the five patients were analysed for their TB-activity. The sera of patients Ja and Gr (first infection) did not block the transmission The sera of patients Ha, Sp and Ro were tested in threefold dilutions Results of the bioassay and ELISA are presented in Table 1 and Figure 1 Only serum of the patient Sp blocked the transmission on admission A maximum TB-titre of ≥81 was obtained in sera of patient Sp over the next 3 weeks and thereafter the titre decreased but significant blocking activity remained until week 83 after admission The sera of patients Ha and Ro developed TB activity in the first and second week respectively after admitted to the hospital which remained positive for at least three months

Antibody reactivity against Pfs230 and Pfs48/45

Sera of patients Ha, Sp and Ro were analysed for the presence of anti-Pfs230 and Pfs48/45 antibody reactivity in competition-ELISAs Table 1 shows the results of competi

TABLE 1. Transmission-blocking activity (R) in the bioassay and reciprocal competition ELISA titres for Pfs230 (epitope I) and epitopes of Pfs48/45 of *P.falciparum* sexual stage parasites in serum samples of 3 gametocyte carriers.

				Epitope of Pfs48/45[&]					
Patient	**Wks**	**R[#]**	**Pfs230**	**I**	**IIa**	**IIb**	**IIc**	**III**	**V**
Ha	adm	<3	<20	160	<20	<20	<20	<20	20
	1	3	<20	160	<20	20	40	20	20
	3	27	<20	1280	160	320	160	160	40
	10	9	<20	640	40	40	40	80	40
	17	9	<20	80	20	40	80	20	40
	53	<3	<20	20	<20	<20	<20	<20	<20
Sp	-2	9	80	160	80	<20	<20	160	320
	adm	≥81	1280	640	640	<20	80	2560	2560
	3	≥81	2560	2560	1280	<20	80	2560	2560
	20	9	1280	640	640	<20	80	1280	1280
	35	9	320	80	160	<20	40	160	160
	83	3	80	40	80	<20	40	80	80
Ro	adm	<3	<20	<20	<20	<20	<20	<20	<20
	1	<3	<20	<20	<20	<20	<20	<20	<20
	2	3	20	20	<20	<20	20	20	<20
	3	3	80	40	<20	<20	40	80	20
	4	nd	80	80	<20	<20	40	80	20
	12	9	320	80	<20	<20	40	160	40

[&] = A NP40 extract of NF54 parasites was used as a source of Pfs230 and Pfs48/45 antigen in all competition ELISAs except for epitope IIa of Pfs48/45 where an extract of 7G8 parasites was used, [#] = reciprocal dilution of the serum with ≥85% TB-activity.

tion on epitope I of Pfs230. Comparable results were found for epitope II and III of Pfs230 (data not shown). The competition-Pfs230 titre of sera from patient Sp and Ro increased and remained positive for at least 83 and 12 weeks respectively. Sera of patient Ha remained negative in all Pfs230 competition-ELISAs despite the presence of gametocytes for a period of approximately 3 weeks.

The results of the competition ELISAs with different epitopes of Pfs48/45 are also shown in Table 1 Patient Ha produces antibodies against all epitopes of Pfs48/45 whereas patient Sp and Ro do not produce competing antibodies against epitope IIb and epitope IIa and IIb respectively A positive competition-ELISA titre to any of the Pfs48/45 epitopes is not always associated with TB-activity. For example in patient Ha a titre of 1.160 against epitope I coincides with no blocking activity in the admission sample, whereas a titre of 1 80 in the sample of week 17 is associated with a TB-titre of 1:9. There is good agreement between TB-activity and the competition-ELISA titre against epitope III of Pfs48/45 (Figure 1). The sera of patients Ja and Gr (first infection) were negative in all competition-ELISAs for both Pfs230 and Pfs48/45.

Antibodies against asexual and sexual stage parasites

The antibody reactivity against asexual- and sexual stage parasites of isolate NF54 were determined by IFA and suspension-IFA (SIFA) in serum samples from patients Ja and Gr (first infection) and patients Ha, Sp and Ro (with a history of malaria)

On admission patient Ja showed an asexual parasite IFA-titre of 1/1280. Patient Gr was negative despite a parasitemia of 1%, but showed a titre of 1/320 one week after start of treatment. The IFA-titre decreased over time but remained detectable for at least 8 weeks (data not shown) Patients Ha, Sp and Ro exhibited IFA-titres against asexual parasites of 1/5120 which persisted for 10 weeks and then started to decline but remained positive for at least 83 weeks in sera of patient Sp (Figure 2)

In the IFA on gametocytes the sera of patients Ja and Gr remained negative whereas the sera of patients Ha, Sp and Ro showed a maximum titre of respectively 1/320, 1/2560 and 1/640. Antibody titres in the gametocyte IFA increased during the time that gametocytes are detectable in the thick smear and decreased slowly thereafter (Figure 2) As shown for patient Ha and Ro, there is no correlation between a positive IFA and TB-activity

The sera of these five patients were further analysed on reactivity with the surface of gametes using the SIFA The sera of patients Ja and Gr were negative whereas the sera of gametocyte carriers Ha, Sp and Ro showed a positive reaction with the surface of the gametes and the SIFA-titres are depicted in Figure 2 As shown in sera from patient Ha and Ro, a positive reaction could be detected in the SIFA without TB-activity as measured

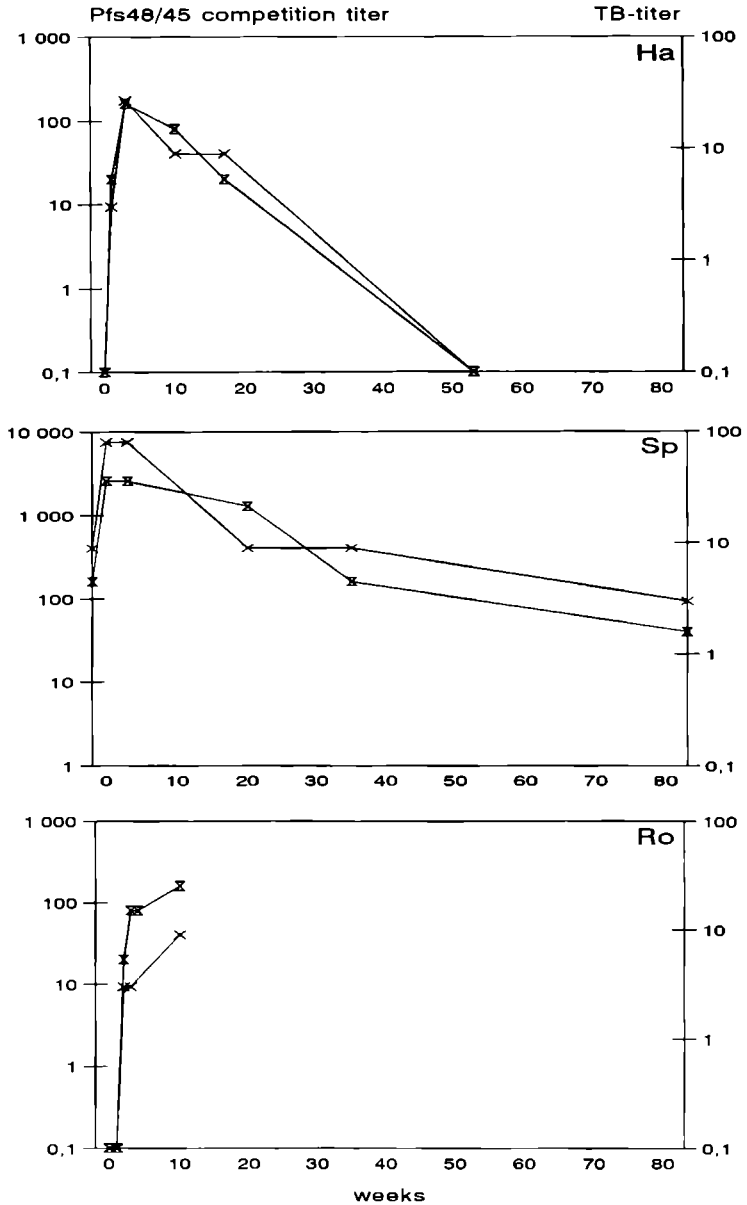


Figure 1: Relationship of competition ELISA values (I) for epitope III of Pfs48/45 and TB-activity ($\geq$85%) (X) in sera from three gametocyte carriers (patient Ha, Sp or Ro) on admission and after treatment.

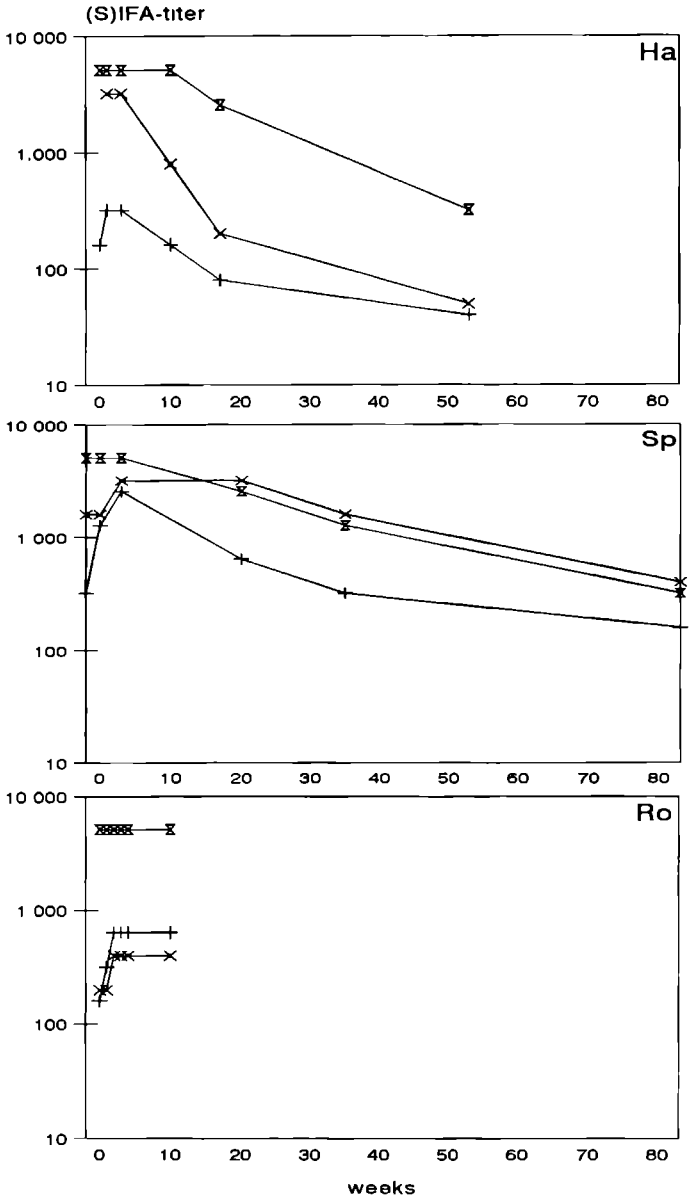


Figure 2: Antibody reactivity against asexual (I) and sexual (+) parasites as detected in the IFA and against gametes (X) using the SIFA in sera from patients Ha, Sp and Ro with a *P.falciparum* malaria history.

in the bioassay A remarkable observation is that the SIFA-titre reach higher values in comparison with the IFA-titre in sera from patient Ha and Sp.

DISCUSSION

This study describes presence, development and persistence of antibody reactivity against asexual and sexual stage parasites and of TB-immunity in five patients with a *P falciparum* malaria infection. In two patients only asexual stage parasites were found, and one patient already had at admission antibodies against trophozoites; the other patient developed such antibodies during and after therapy. Both were negative in antibody tests performed with antigens of the sexual stages This absence of circulating antibodies against the sexual stages might indicate that these patients had been infected for the first time

Antibody responses to gametocytes antigens are observed after exposure to *P falciparum* [5,6,7,8,11,18,19,22,24] Shute and Maryon [22] studied patients with a primary infection and observed that 45% did not develop gametocytaemia detectable in blood smears, whereas Ong *et al.* [11] found that 12 out of 14 patients with a primary infection developed sexual stage antibodies as detected by IFA but observations on gametocytes in blood smears were not included in this study. Also Mendis *et al.* [10] reported about rapidly induced antibody reactivity against gametocytes, even with TB-activity, in sera from gametocyte carriers during primary *P vivax* infections in Sri Lanka Shute and Maryon [22] indicated that only when parasite patency persisted for a period of three weeks or more the chances of the detection of a gametocytaemia detection were high Patients Ja and Gr indicated that they visited the hospital within a week after the start of their illness, and this may explain the absence of a gametocytaemia in these patients

The (S)IFA analyses show that in patients with a malaria history SIFA-titres are higher than IFA-titres for as yet unknown reasons Since a positive SIFA indicates antibody reactivity to the surface of activated extra-erythrocytic gametocytes or free gametes/-zygotes it is a good candidate for correlation with TB-immunity. Premawansa *et al.* [15] found a strong correlation between the intensity of response to gamete surface antigens by SIFA and TB-effects of these sera from Sri Lanka. Our data show, however that a) SIFA titre may be high before TB-immunity has been developed which is clear in the first samples of patients Ha and Sp and b) the SIFA titre does not correlate with the TB-titre. In view of cross reactivity between different stages due to common molecules and cross-reactions of antibodies stimulated by different antigens as observed by Saul *et al* [21] it is advisable to analyse antibody reactivity and TB-immunity at the epitope level

Patients Ha and Ro were admitted with a gametocytaemia, which could effectively be

transmitted to mosquitoes in the presence of own plasma or after replacement by normal serum, indicating that these patients had no TB-activity in their serum. TB-activity developed in time in these patients and persisted after disappearance of the gametocytes. It should be noted that peak antibody titres were observed approximately three weeks after admission, which may be approximately five weeks or more after the first complaints.

Development of TB-immunity coincided with development of antibodies that reacted with epitopes on Pfs48/45 as detected in competition-ELISAs and there was a good agreement between TB-immunity and competition-ELISA titres particularly to epitope III. Ong *et al.* [11] found with epitope III the most competition reactions in comparison with epitope IIc and V in sera from patients with a primary infection or with a malaria history. Together these data suggest that in these patients Pfs48/45 could be a target of TB-immunity. In two studies Graves *et al.* [5,6] could not detect a correlation between TB-immunity and competition of antibodies for binding to epitope III of Pfs48/45 in a panel of Papua New Guinea sera. In a later study Graves *et al.* [8] found a significant association between TB-activity and recognition of epitope IIa. We have previously decribed a good correlation (100% specificity and 75% overall relative sensitivity) between TB-activity using a feeder assay with NF54 *P.falciparum* parasites and reactivity in Pfs48/45 competition-ELISAs [20]. The data described here largely confirm this correlation except for epitope I. Sera collected from patients with a malaria attack >6 months ago were positive in the competition ELISA only against epitope I of Pfs48/45 and negative in the TB-assay (data not shown). It seems, that antibody reactivity over epitope I of Pfs48/45 is predominant against the other epitopes and does not associate with TB-activity.

A remarkable observation is that patient Ha did not show antibody reactivity against Pfs230 in competition-ELISAs including several epitopes, suggesting that in this patient Pfs230 may not be the target of TB-immunity.

The gametocytaemia at admission and during therapy is not directly related to the TB-titre or the titres of the competition-ELISAs observed later on. The gametocyte load before admission and particularly the exposure to gametocytes at previous infections may play an important role in these parameters as was also suggested in a study by Mendis *et al.* [10] with respect to *P. vivax* infections.

Titres in all antibody tests against both asexual and sexual stage parasites were highest in patient Sp. This may be explained by recurrent parasitemias during his stay as an expatriate for a period of 30 years with repeated malaria infections or by the recrudescence. Taylor *et al.* [25] hypothesized that repeated parasitemias would result in increased antibody titres and/or increased antibody quality (i.e. affinity, avidity, isotype, etc). The higher titres may therefore be the result of a specific boosting of a previous immunologic

response

A remarkable observation is the long period of TB-immunity in the 3 patients with a malaria history after radical cure, which persist until week 83 for patient Sp The long period of TB-immunity observed in these patients in the absence of the parasite is at variance with a common view and so encouraging with regard to feasability of future TB vaccine based on antigens of the sexual stages of the parasite, Pfs48/45 in particular, as an additional tool in malaria control

ACKNOWLEDGEMENTS

We are grateful to Theo Arens for reading thick smears and detection of gametocyte carriers, to Arianne Huisman and Geertjan van Gemert for parasite cultures and transmission experiments, to JHETh Meuwissen for his critical comments during the preparation of the manuscript The generous gift of various mAbs by Dr R Carter, Division of Biological Sciences, Edinburgh, England and Prof Dr G Targett, London school of Hygiene & Tropical medicine, England is thankfully acknowledged Thanks also go to the medical and laboratory staff of Nijmegen Hospital who where very supportive in the study

This research was supported by the Dutch Ministry for Development Co operation (DGIS/SO) contract nr NL002701

REFERENCES

1 CARTER R, BUSHELL G, SAUL A, GRAVES PM & KIDSON C (1985) Two apparently nonrepeated epitopes on gametes of *P falciparum* are targets of transmission blocking antibodies *Infection and Immunity* **50**, 102-106

2 CARTER R, KUMAR N, QUAKYI IA, GOOD M, MENDIS K, GRAVES P & MILLER L (1988) Immunity to sexual stages of malaria parasites *Progess of Allergy* **41**, 193 214

3 CARTER R, GRAVES PM, QUAKYI IA & GOOD MF (1989) Restricted or absent immune responses in human populations to *Plasmodium falciparum* gamete antigens that are targets of malaria transmission-blocking antibodies *Journal of Experimental Medicine* **169**, 135-147

4 FOO A, CARTER R, LAMBROS C, GRAVES P, QUAKYI I, TARGETT G, PONNUDURAI T & LEWIS G (1991) Conserved and variant epitopes of target antigens of transmission-blocking antibodies among isolates of *P falciparum* from Malasia *American Journal of the Tropical Medicine and Hygiene* **44**, 623-631

5 GRAVES PM, CARTER R, BURKOT TR, QUAKYI IA & KUMAR N (1988a) Antibodies to *P falcipa rum* gamete surface antigens in Papua New Guinea sera *Parasite Immunology* **10**, 209 218

6 GRAVES PM, WIRTZ RA, CARTER R, BURKOT TR, LOOKER M & TARGETT GAT (1988b) Naturally occuring antibodies to an epitope on *P falciparum* gametes detected by monoclonal antibody based competitive Enzyme-linked Immunosorbend Assay *Infection and Immunity*, **56**, 2818-2821

7 GRAVES PM, DOUBROVSKY A, CARTER N & BECKERS P (1990) High frequency of antibody response to *P falciparum* gametocyte antigen during acute malaria infections in Papua New Guinea highlanders *American Journal of Tropical Medicine and Hygiene* **44**, 515-520

8 GRAVES PM, DOUBROVSKY A, SATTABONGKOT J & BATTISTUTTA D (1992) Human antibody responses to epitopes on the *P falciparum* gametocyte antigen Pfs48/45 and their relationship to infectivity of gametocyte carriers *American Journal of Tropical Medicine and Hygiene* **46**, 711-719

9 KUMAR N & CARTER R (1984) Biosynthesis of the target antigens of antibodies blocking transmission of *P falciparum* *Molecular and Biochemical Parasitology* **13** 333-342

10 MENDIS KN, MUNESINGHE YD, DE SILVA YNY, KERAGALLA I & CARTER R (1987) Malaria transmission-blocking immunity induced by natural infections of *Plasmodium vivax* in humans *Infection and Immunity* **55** 369-372

11 ONG CSL, ZHANG KY, EIDA SJ, GRAVES PM, DOW C, LOOKER M, ROGERS NC, CHIODINI PL & TARGETT GAT (1990) The primary antibody response of malaria patients to *P falciparum* sexual stage antigens which are potential transmission blocking vaccine candidates *Parasite Immunology* **12**, 447-456

12 PONNUDURAI T, LENSEN AHW, LEEUWENBERG ADEM & MEUWISSEN JHETH (1982) Cultivation of fertile *P falciparum* gametocytes in semi-automated systems 1 Static cultures *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76**, 812-818

13 PONNUDURAI T, VAN GEMERT GJA, BENSINK T, LENSEN AHW & MEUWISSEN JHETH (1987) Transmission blockade of *P falciparum* its variability with gametocyte numbers and concentration of antibody *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 491-493

14 PONNUDURAI T, LENSEN AHW, VAN GEMERT GJA, BENSINK MPE, BOLMER M & MEUWISSEN JHETH (1989) Infectivity of cultured *P falciparum* gametocytes to mosquitoes *Parasitology* **98**, 165-173

15 PREMAWANSA S, GAMAGE-MENDIS A, PERERA L, BEGARNIE S MENDIS K & CARTER R (1994) *Plasmodium falciparum* transmission blocking immunity under conditions of low endemicity as in Sri Lanka *Parasite Immunology* **16**, 35-42

16 READ D, LENSEN AHW, BEGARNIE S, HALEY S, RAZA A & CARTER R (1994) Transmission blocking antibodies against multiple, non-variant target epitopes of the *Plasmodium falciparum* gamete surface antigen Pfs230 are all complement-fixing *Parasite Immunology* **16**, 511-519

17 RENER J, GRAVES PM, CARTER R, WILLIAMS JL & BURKOT TA (1983) Target antigens of transmission-blocking immunity on gametes of *P falciparum* *Journal of Experimental Medicine* **158**, 976-981

18 ROEFFEN W, LENSEN T, MULDER B, TEELEN K, SAUERWEIN R VAN DRUTEN J, ELING W, MEUWISSEN JHETH & BECKERS PJA (1995a) A comparison of transmission blocking activity with reactivity in a *P falciparum* 48/45 kD molecule specific competition ELISA *American Journal of the Tropical Medicine and Hygiene* **52**, 60-65

19 ROEFFEN W, BECKERS PJA, TEELEN K, LENSEN T, SAUERWEIN RW, MEUWISSEN JHETH & ELING W (1995b) *P falciparum* A comparison of the activity of Pfs230 specific antibodies in an assay of transmission-blocking immunity and specific competition ELISA *Experimental Parasitology* **80**, 15 26

20 ROEFFEN W, MULDER B, TEELEN K, BOLMER M, ELING W, TARGETT GAT, BECKERS PJA & SAUERWEIN R (1996) Association between anti Pfs48/45 reactivity and *P falciparum* transmission-blocking activity in sera from Cameroon *Parasite Immunology* **18**, 103-109

21 SAUL A, LORD R, JONES G, GEYSEN HM, GALE J & MOLLARD R (1990) Cross reactivity of antibody against an epitope of the *Plasmodium falciparum* second merozoite surface antigen *Parasite immunology*, **11**, 593-601

22 SHUTE PG & MARYON M 1951 A study of gametocytes in a west african strain of *Plasmodium falciparum Transactions of the Royal Society of Tropical Medicine and Hygiene* **44**, 421 438

23 TARGETT GAT, HARTE PG, EIDA S, ROGERS NC & ONG CSL (1990a) *P falciparum* sexual stage antigens immunogenecity and cell mediated resposes *Immunological letters* **25**, 77 - 82

24 TARGETT GAT (1990b) Immunity to sexual stages of human malaria parasites Immune modulation during natural infections, antigenic determinants, and the induction of transmission-blocking immunity *Scandinavian Journal of Infection Diseases* Supplement **76**, 79-88

25 TAYLOR 1989

26 VERMEULEN AN, PONNUDURAI T, BECKERS PJA, VERHAVE JP, SMITS MA & MEUWISSEN JHETH (1985) Sequential expression of antigens on sexual stages of *P falciparum* accessible to transmission blocking antibodies in the mosquito *Journal of Experimental Medicine* **162,** 1460-1476

CHAPTER 8

Summary and General Discussion.

SUMMARY AND GENERAL DISCUSSION

The first successful induction of transmission-blocking (TB) immunity was reported by Gwadz [12] and Carter & Chen [1] in 1976 for the avian malaria parasite *P gallina ceum* Subsequently Carter *et al* [2] showed that the target proteins were present on the surface of both male and female gametes of *P gallinaceum*

Antibodies against *P falciparum* gametocytes were found in sera from malaria endemic regions [26] and the ability of such antibodies to block transmission was shown by Meuwissen *et al* [17] and Graves *et al* [8] TB-antibodies can apparently be induced during the natural course of *P falciparum* or *P vivax* malaria infections [4,6,8,9,chapter II] Remarkably, antibody concentrations are highest in individuals with an incidental exposure, whereas people with frequent exposure exhibit lower antibody responses [3,16]

The target antigens of this immunity are considered to be membrane-bound proteins expressed on the surface of gametes when released from their erythrocytes in the mosquito midgut after ingestion of a bloodmeal Gametocytes of *P falciparum* synthesize Pfs230 and Pfs48/45 and express these proteins on the surface of freshly released gametes and zygotes [15,22,25] Most epitopes of these sexual stage antigens that react with natural antibodies are conformation dependent As these proteins are potential candidates for a TB-vaccine, it is important that gametocytes boost immunity to these proteins during a natural infection

Most individuals exposed to a *P falciparum* infection with a gametocytaemia develop antibody responses to gametocyte antigens [4,7,18, chapter IV] The prevalence of natural antibodies to Pfs230 and Pfs48/45 is dependent of age and geographical origin of the studied population [21] Anti Pfs230 antibodies have been reported in 40 70% of the sera from individuals living in malaria endemic regions [7,24] and in up to 100% of gametocyte carriers [chapter II] Natural anti-Pfs48/45 antibodies are found in 15-87% of sera from malaria endemic areas [11,23] Riley *et al* [23] found in a study in The Gambia that only 50% of malaria-exposed individuals exhibited cellular or humoral responses to Pfs48/45 but according to their opinion responsiveness could not be explained by MHC class II restriction Other explanations for unresponsiveness must be looked for

The objectives of the studies described in this thesis were a) the development of serological tests to study the prevalence of antibodies against Pfs230 and/or Pfs48/45 in sera from naturally infected individuals and b) to study the association between the presence of these antibodies and the capacity of sera to block *P falciparum* transmission

At least five different epitopes of Pfs230 can be detected using a panel of mouse anti Pfs230 mAbs All these mAbs of the complement fixing IgG2a/IgG2b subclasses block transmission by lysis of released gametes, whereas none of the IgG1 mAbs (no capacity to

fix complement) show this activity independent of their epitope specificity [chapter III]. Thus, both lytic TB-mAbs and non-blocking mAbs can compete for binding to a given epitope [chapter II and III] Complement dependent lysis of gametes and zygotes *in vitro* by anti-Pfs230 mAbs has also been described for *P.gallinaceum* parasites by Quakyi *et al.* [20] and Kaushal *et al.* [13]

Immunoprecipitation experiments indicated that the presence of anti-Pfs230 antibodies correlate with TB-activity in sera from an endemic area in Papua-New Guinea [8] whereas no correlation was found in sera from Sri Lanka [19]. TB-activity was observed in 40% of the sera from a group of gametocyte carriers from Cameroon; all these sera immunoprecipitated Pfs230 [chapter II] and reacted in isotype specific Pfs230 ELISAs [data not shown-] Furthermore, only 10% of these sera exhibited activity in one of the anti-Pfs230 competition-ELISAs (C230-ELISAs) A possibly explanation for different reactivities in these assays may be the low affinity of these natural anti-Pfs230 antibodies in comparison with that of competing mAbs Our serological data neither show a correlation between immunoprecipitation of Pfs230 and TB-activity nor between TB-activity and titers of C230-ELISAs [chapter II,IV and VII].

None of the sera from gametocyte carriers with TB-activity exhibited a complement dependent lysis of gametes as described for the anti-Pfs230 mAbs Furthermore, all these sera are positive in the isotype specific anti-Pfs230 ELISAs. Interestingly, IgG1 and IgG3 are prominent isotypes reacting with Pfs230 in these sera, while IgG2 and IgG4 are low or absent [data not shown]; notwithstanding the fact that these isotypes are the principle subclasses that fix complement, no lysis of gametes was observed with these sera The discrepancy between lytic reactivity of complement binding mouse anti-Pfs230 mAbs and the corresponding natural human antibodies that lack lytic activity remains to be explained

In conclusion, the presence of anti-Pfs230 antibodies in natural sera, even of complement fixing isotypes, is not associated with TB-activity. These data do not support an important role for anti-Pfs230 antibodies in natural TB-activity Discrepancies with other studies may be related to differences in studied geographical region and methods used.

A comparison analysis was made between the titre in a anti-Pfs48/45 competition-ELISA (C45-ELISA) with a TB-mAb (32F3; epitope I) and TB-activity (bio-assay) in serum samples of a group of A) European expatriates with incidental exposure to malaria, B) Cameronian gametocyte carriers and C) randomly selected schoolchildren from malaria endemic areas in Cameroon Of all serum samples with TB-activity, 47% of group A, 52% of group B and 20% of group C were positive in the C45-ELISA; the overall sensitivity was 44% with a relative specificity of 94% implying a fair to good agreement [chap-

ter V]. Serum samples that were positive in more than one epitope-specific C45-ELISA were all positive in the TB-assay Interestingly, reactivity in the epitope I specific C45-ELISA did not correlate with TB-activity in a) one out of three serum samples of two European expatriates (different from group A) taken before occurence of TB-immunity [chapter VII] and b) in seven sera out of another group of eight European expatriates with no TB-activity who experienced their last infection six months or longer before sampling [data not shown] Reactivity to epitope I may therefore be associated with TB-activity only during a part of the period of the anti-Pfs48/45 antibody response

Sera from another group of Cameronian gametocyte carriers were analysed using C45-ELISAs for five different epitopes of Pfs48/45 [chapter VI]. Comparison of TB-activity and competition reactivity for five epitopes of Pfs48/45 shows a relative specificity of 100% and a relative sensitivity of 25%, 39%, 61%, 54% and 57% for respectively epitope I, IIb, IIc, III and V with an overall sentitivity of 75% Ong *et al* [18] found in sera from malaria patients, with either a primary infection or with a malaria history a higher frequency of competition reactivity for epitope III than for epitope IIc or V but correlation with TB-activity was not determined in these sera. Graves *et al* [8] found no correlation between serologic reactivity to Pfs48/45 and TB-activity in a panel of sera from Papua New Guinea using immunoprecipitation of Pfs48/45 and competition of antibodies with mAb epitope III of Pfs48/45 [7] Discrepancies between this and our studies may be related to differences in immune reactivity in populations from geographically different areas

Studies on different isolates of *P.falciparum* showed that antibody reactivity to epitopes I, IIb and III were comparable while variations existed in reactivity to epitope IIa and IIc [chapter VI]. This result is in line with observations by Foo *et al* [5] who showed that epitope I, IIb, III and IV are conserved and epitope IIa and IIc are allelic forms of Pfs48/45. Competition reactivity for both epitope IIa and IIc was found in sera from Dutch expatriates and in 3 out of 11 sera from Cameronian gametocyte carriers [chapter VI]. Drakeley *et al.* [personal communication] found mixed infections of IIa/IIc in the Gambia, Cameroon, Tanzania and Thailand whereas only IIa was detected in Brazil using the polymerase chain reaction (PCR) technique [14]. Our data confirm antibody reactivity against both epitope IIa and IIc parasatised infections in some sera from Cameroon [chapter VI] Preliminary data suggest that TB-activity is probably not dependent on reactivity against the IIa or IIc allel of Pfs48/45 [chapter VII]

In a longitudinal study (up to 80 weeks) in *P.falciparum* infected expatriates from malaria endemic areas, development of TB-immunity correlated with C45-ELISA titers particularly to epitope III. The titres in these sera (up to 1/2560) were significantly higher

than those in gametocyte carriers from Cameroon (up to 1/80) [chapter VI and VII] This corresponds with reactivity observed respectively after incidental or continuous exposure as described by Carter *et al* [3] and Lulat [16] Graves *et al* [10] found four distinct patterns in time-course responses to four epitopes of Pfs48/45 using the C45-ELISA of endemic sera, marking the complexity of the antibody response to Pfs48/45 These and our findings indicate that studies on a relation between serologic reactivity and TB-activity should be carried out at the epitope level

Interestingly, not only titres but also the relative specificity and sentivity of epitope I and epitope III specific C45-ELISAs differ between the groups European expatriates and gametocyte carriers from Cameroon The relative sensitivities for epitope I in the C45-ELISA were 71% (n=53) and 35% (n=174) with relative specificities of 71% and 95% and for epitope III, relative sensitivities were 94% (n=26) and 54% (n=52) with corresponding relative specificities of 90% and 100% respectively in the expatriates and gametocyte carriers. These data show that overall the C45-ELISA for epitope III is a better marker than that for epitope I for the comparison with TB-activity in serum samples

Another interesting observation in our study with Europian expatriates was the period of persistence of TB-immunity ranging from 50 to 80 weeks after the last exposure to gametocytes This long period of TB-immunity in the absence of the parasite is encouraging for the development of a TB-vaccine based on antibody reactivity against gametocyte antigens and against Pfs48/45 in particular. No data are available on persistence of established TB-immunity against *P.falciparum* in endemic areas Mendis *et al* [6] found that loss of TB-activity can occur in the absence of re-exposure to *P.vivax* malaria in a period of four months

There is no consistency in the relationship between antibody titers against antigens of the sexual stages and TB-activity in different studies. This may be due to differences in endemicity of malaria in the groups studied. Studies on TB-activity and reactivity in C45-ELISAs involving all available epitopes in populations which differ in age, geographic origin and exposure to different transmission intensities may clarify this point. In addition, studies on strain specificity of TB-immunity and corresponding C45-ELISAs need to be expanded Such studies may reveal whether or not TB-activity under field conditions is strain, molecule and/or epitope specific These data are needed to define a role for Pfs48/-45 in the epidemiology of malaria transmission Serological tests as described in this thesis, may contribute to a better understanding of the epidemiology of malaria transmission and may indirectly help to find new ways for control of malaria.

The individual differences and the duration of individual immune responses to the sexual stage antigens in relation to exposure, are of great importance for the ultimate

effect of a TB vaccine

REFERENCES

1 CARTER R & CHEN DH (1976) Malaria transmission blocked by immunization with gametes of the malaria parasite *Nature* **293** 57-60

2 CARTER R, GWADZ RW & GREEN I (1979) *P gallinaceum* transmission blocking immunity in chickens II The effect of antigamete antibodies *in vitro* and *in vivo* and their elaboration during infection *Exp Paras* **47** 194-208

3 CARTER R, GWADZ RW & GREEN I (1981) Naturally acquired immunity and anti malarial antibodies in relation to infectivity to mosquitoes in endemic *P falciparum* malaria Immuno diagnostic techniques in malaria UNDP/WORLD BANK/WHO

4 CARTER R, GRAVES PM, QUAKYI IA & GOOD MF (1989) Restricted or absent immune responses in human populations to *Plasmodium falciparum* gamete antigens that are targets of malaria transmission-blocking antibodies *J Exp Med* **169** 135 147

5 FOO A, CARTER R, LAMBROS C, GRAVES P, QUAKYI I, TARGETT G, PONNUDURAI T & LEWIS G (1991) Conserved and variant epitopes of target antigens of transmission blocking antibodies among isolates of *P falciparum* from Malasia *Am J Trop Med Hyg* **44** 623 631

6 GAMAGE-MENDIS R, RAJAKARUNA J, CARTER R & MENDIS K (1992) Transmission blocking immunity to human *P vivax* malaria in an endemic population in Kataragama, Sri Lanka *Parasite Immunol* **14** 385 396

7 GRAVES PM, CARTER R, BURKOT TR, QUAKYI IA & KUMAR N (1988) Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera *Parasite Immunol* **10** 209 218

8 GRAVES PM, WIRTZ RA, CARTER R, BURKOT TR, LOOKER M & TARGET GAT (1988) Naturally occurring antibodies to an epitope on *Plasmodium falciparum* gametes detected by monoclonal antibody based competitive enzyme-linked immunosorbent assay *Inf Immun* **56** 2818 2821

9 GRAVES PM, DOUBROUSKY A, CARTER R, EIDA S & BECKERS P (1990) High frequency of antibody response to *Plasmodium falciparum* gametocyte antigens during acute malaria infections in Papua New Guinea highlanders *Am J Trop Med Hyg* **42** 515-520

10 GRAVES PM, DOUBROUSKY A & BECKERS P (1991) Antibody responses to *Plasmodium falciparum* gametocyte antigens during and after malaria attacks in schoolchildren from Madang Papua New Guinea *Parasite Immunol* **13** 291 299

11 GRAVES PM, DOUBROVSKY A, SATTABONGKOT J & BATTISTUTTA D (1992) Human antibody responses to epitopes on the *Plasmodium falciparum* gametocyte antigen Pfs48/45 and their relationship to infectivity of gametocyte carriers *Am J Trop Med Hyg* **46** 711 719

12 GWADZ RW (1976) Malaria succesful immunization against the sexual stages of *Plasmodium gallinaceum* *Science* **193**:1150-1151

13 KAUSHAL DC, CARTER R, RENER J, GROTENDORST CA, MILLER LH & HOWARD RJ. (1983) Monoclonal antibodies against surface determinants on gametes of *Plasmodium gallinaceum* block transmission of malaria parasites to mosquitoes. *J Immunol* **131**:2557-2562.

14 KOCKEN CHM, MILEK RLB, LENSEN THW, KASLOW DC, SCHOENMAKERS JGG & KONINGS RNH. (1995) Minimal variation in the transmission-blocking vaccine candidate Pfs48/45 of the human malaria parasite *P. falciparum*. *Mol Biochem Parasit* **69:**115-118

15 KUMAR N & CARTER R. (1984) Biosynthesis of the target antigens of antibodies blocking transmission of *P. falciparum*. *Mol Biochem Parasit* **13:**333-342.

16 LULAT A. (1993) A study of humoral and cell-mediated immune responses to *P.falciparum* malaria gametocyte antigens in a rural Gambian population. PhD Thesis, University of London.

17 MEUWISSEN JHETH, PONNUDURAI T & VERMEULEN AN. (1985) Candidate antigens: Studies on the development of a *P.falciparum* malaria vaccine for the induction of transmission-blocking immunity. In: *Proceedings of the Asia and Pacific Conference on Malaria,* Siddiqui W.A. (Editor). Hawaii: University of Hawaii, pp. 133-142.

18 ONG CSL, ZHANG KY, EIDA SJ, GRAVES PM, DOW C, LOOKER M, ROGERS NC, CHIODINI PL & TARGETT GAT. (1990) The primary antibody response of malaria patients to *P. falciparum* sexual stage antigens which are potential transmission blocking vaccine candidates. *Parasite Immunol* **12:**447-456.

19 PREMAWANSA S, GAMAGE-MENDIS A, PERERA L, BEGARNIE S, MENDIS K & CARTER R (1994) *Plasmodium falciparum* transmission-blocking immunity under conditions of low endemicity as in Sri Lanka. *Parasite Immunology* **16:**35-42.

20 QUAKYI IA, CARTER R, RENER J, KUMAR N, GOOD M F. & MILLER LH. (1987). The 230-kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmisssion-blocking antibodies. *J Immunol* **139**:4213-4217.

21 QUAKYI IA, OTOO LN, POMBO D, SUGARS LY, MENON A, DEGROOT AS, JOHNSON A, ALLING D, MILLER LH & GOOD MF. (1989) Differential non-responsiveness in human of candidate *Plasmodium falciparum* vaccine antigens. *Am J Trop Med Hyg* **41**.125-134

22 RENER J, GRAVES PM, CARTER R, WILLIAMS JL & BURKOT TA. (1983) Target antigens of transmission-blocking immunity on gametes of *P. falciparum*. *J Exp Med* **158:** 976-981.

23 RILEY EM, ONG CS, OLERUP O, EIDA S, ALLEN SJ, BENNETT S, ANDERSSON G AND TARGETT GA. (1990) Cellular and humoral immune responses to Plasmodium falciparum gametocyte antigens in malaria immune individuals. Limited response to the 48/45-kilodalton surface antigen does not appear to be due to MHC restriction. *J Immunol* **144**:4810-4816

24 RILEY EM, BENNETT S, JEPSON A, HASSANKING M, WHITTLE H, OLERUP O & CARTER R. (1994) Human antibody responses to Pfs230, a sexual stage-specific surface antigen of *Plasmodium Falciparum:* non-responsiveness is a stable phenotype but does not appear to be genetically regulated. *Parasite Immunol* **16**:55-62

25 VERMEULEN AN, PONNUDURAI T, BECKERS PJA, VERHAVE JP, SMITS MA & MEUWISSEN JHETH. (1985) Sequential expression of antigens on sexual stages of *P. falciparum* accessible to transmission blocking antibodies in the mosquito. *J Exp Med* **162:**1460-1476.

26 VOLLER A & BRAY RS (1962) Fluorescent antibody staining as a measure of malarial antibody *Proc Soc Exp Biol Med* **110** 907-910

CHAPTER 9

SAMENVATTING.

SAMENVATTING

In de tropen en subtropen is malaria een van de meest voorkomende ziekten. Jaarlijks maken wereldwijd ongeveer 300-500 miljoen mensen de infectie door Als gevolg daarvan sterven per jaar ca. 2 miljoen mensen, voornamelijk jonge kinderen en dat aantal is weer aan het toenemen, onder andere omdat geneesmiddelen hun werkzaamheid verliezen
Malaria wordt veroorzaakt door een eencellige parasiet behorende tot de protozoa van het genus Plasmodium Van de tientallen Plasmodium soorten komen er vier bij de mens voor. Levensbedrijgend is *Plasmodium falciparum.*

In het menselijk lichaam bevinden de parasieten zich gedurende het grootste deel van de tijd in de rode bloedcellen waarin een ongeslachtelijke voortplanting plaats vindt. In enkele parasieten wordt via een nog onbekend mechanisme de ontwikkeling van geslachtelijke stadia geinduceerd. Deze gametocyten rijpen verder uit en blijven in de rode bloedcel tot een vrouwelijke mug (van het genus Anopheles) een bloedmaal neemt. In de maag van de mug komen de gameten uit de gametocyten vrij en heeft een geslachtelijke voortplanting plaats. Hieruit ontstaan weer nieuwe vormen van de parasiet, die na circa tien dagen in de speekselklieren van de mug nestelen. Bij een steek van deze geinfecteerde mug komen de parasieten, sporozoieten genaamd, in de bloedbaan van de mens en zorgen voor een infectie.

Een van de mogelijkheden ter bestrijding van malaria is de ontwikkeling van een vaccin. Minstens drie vaccin typen zouden hiervoor in aanmerking kunnen komen; a) een sporozoieten vaccin kan de gastheer beschermen tegen het binnendringen van de parasiet in de levercellen en daarmee de verdere ontwikkeling van de parasiet blokkeren, b) een merozoieten vaccin kan alleen de asexuele cyclus onderbreken en zo het ontstaan van de ziekte-verschijnselen voorkomen en c) een vaccin tegen sexuele stadia kan de verdere ontwikkeling van de parasiet in de mug blokkeren en daardoor de transmissie van de parasiet van de mug op de mens voorkomen

Op de afdeling medische microbiologie, sectie parasitologie van het academisch ziekenhuis in Nijmegen wordt gewerkt aan de ontwikkeling van een transmissie-blokkerend (TB) vaccin Het startpunt in dit promotie onderzoek betrof de observatie dat bij mensen, die in malaria gebieden leven, antistoffen voorkomen die met de sexuele stadia van de parasiet reageren en dat sommige daarvan de ontwikkeling van de parasiet in de malaria mug kunnen blokkeren Er zijn monoclonale antistoffen (mAbs) ontwikkeld, die zich binden aan de oppervlakte eiwitten van de sexuele stadia van de parasiet in de mug en die daarbij de ontwikkeling van de parasiet blokkeren

Met behulp van transmissie blokkerende antistoffen zijn oppervlakte eiwitten van de

parasiet geïdentificeerd. Het bleek dat oppervlakte eiwitten die op de sexuele stadia herkend worden, verdeeld zijn over twee ontwikkelings fasen De eiwitten van de eerste vroege fase (Pfs230 en Pfs48/45) komen direct na activering van de gametocyten voor op het oppervlak van de gameten/zygoten en antilichamen hiertegen voorkomen waarschijnlijk de bevruchting van de vrouwelijke gameet. In de tweede fase, vanaf ongeveer drie uur na de aktivatie van de gametocyten en vorming van zygoten, komt eiwit Pfs25 aan het oppervlakte van de gameten/zygoten, wanneer Pfs230 en Pfs48/45 grotendeels zijn afgestoten Bij de mens zijn alleen antistoffen gevonden tegen de eiwitten van eerste vroege fase en niet van tweede fase.

In de afgelopen 10 jaar is immunologisch onderzoek gedaan naar de antistoffen die zich binden aan deze oppervlakte-eiwitten van de parasiet. Het in dit proefschrift beschreven onderzoek gaat over antistoffen die gericht zijn tegen de vroege fase 1 eiwitten, Pfs230 en Pfs48/45.

In een laboratoriumtest (TB-test) voor de bepaling van transmissie-blokkerende antistoffen worden *in vitro* gekweekte gametocyten, in aanwezigheid van antistoffen gevoed aan vrouwelijke muggen. Zeven dagen later wordt het aantal oocysten in de muggemaag microscopisch geteld en het blokkerend vermogen van het te testen serum bepaald ten opzichte van een controle serum. Deze TB-test is een tijdrovende, ingewikkelde en dure test die veel infrastrukturele voorzieningen vergt (bv. parasieten- en muggenkweek) en waarmee een beperkt aantal sera kan worden getest. Daarom wordt gezocht naar een makkelijk te hanteren test die de aanwezigheid van transmissie-blokkerende antistoffen kan voorspellen

De doelstellingen van de studies die in dit proefschrift zijn beschreven zijn als volgt:

1) de ontwikkeling van serologische testen, die de aanwezigheid van anti-Pfs230- en anti-Pfs48/45 antistoffen in sera van malaria patienten aantonen,
2) te bepalen in hoeverre deze antistoffen correleren met de capaciteit van het serum om de transmissie te blokkeren.

De ontwikkeling en activiteit van anti-Pfs230 mAbs wordt in hoofdstuk 2 beschreven Het blijkt dat mAbs gericht tegen Pfs230 van het isotype IgG2a of IgG2b, in staat zijn om complement te binden en de parasiet te vernietigen, zo wordt de transmissie geblokkeerd MAbs tegen Pfs230 van het isotype IgG1 kunnen dit niet Met deze mAbs zijn ELISA's ontwikkeld die een indruk geven van het epitopencomplex van Pfs230. Er blijken minimaal 5 epitopen te zijn, waarmee zowel blokkerende- als niet blokkerende mAbs in competitie met elkaar reageren. In hoofdstuk 3 wordt aangetoond dat in aanwezigheid van complement een niet-blokkerende mAb blokkerend wordt als het isotype "switcht" van IgG1 naar IgG2b of IgG2a Hieruit kunnen we concluderen dat complement bindende

mAbs gericht tegen verschillende epitopen van Pfs230 de transmissie kunnen blokkeren

Anti-Pfs230 antistoffen komen voor in 40 70% van de sera van mensen die in endemische malaria gebieden wonen en in alle mensen die uit deze gebieden met *P falciparum* gametocyten in hun bloed terugkeren (hoofdstuk 2) Met de ontwikkelde Pfs230 competitie ELISA's is de reactiviteit van antistoffen in humane sera bepaald Er kon geen associatie gevonden worden tussen de reactiviteit in de competitie-ELISA's en het blokkerend effect van het serum in de TB-test In een inleidend experiment is in veldsera het isotype van anti-Pfs230 antistoffen bepaald Hieruit bleek dat complement fixerende antistoffen aan Pfs230 binden, maar een transmissie blokkerende activiteit in dergelijke sera was niet complement afhankelijk Mogelijk is de affiniteit van natuurlijke anti Pfs230 antistoffen laag in vergelijking met die van de mAbs Dit zou erop wijzen dat de antistoffen tegen Pfs230 - tenminste in bepaalde groepen malaria patienten - waarschijnlijk niet steeds tot blokkade van de transmissie leiden

Anti-Pfs48/45 antistoffen komen voor in 15-87% van de sera van mensen die in malaria endemische gebieden wonen In het eerste onderzoek werd het transmissie blokkerend vermogen van een serum vergeleken met de activiteit in competitie ELISA's (hoofdstuk 4) In vervolg-studies wordt een vergelijkende analyse gebruikt om de overeenkomst tussen de activiteit in competitie-ELISA's en de TB test te bepalen in sera van a) reizigers uit Europa, b) gametocytendragers en c) schoolkinderen uit een endemisch malaria gebied in Kameroen De gecombineerde resultaten van al deze bepalingen laat een goede overeenkomst zien tussen de resultaten verkregen met de competitie-ELISA en de TB test Met deze competitietesten kan 44% van de transmissie blokkerende sera worden opgespoord, als de reaktiviteit tegen één epitoop bekeken wordt en 50% indien twee epitopen in de analyse worden betrokken (hoofdstuk 4 en 5) In vervolg onderzoek worden zes verschillende anti-Pfs48/45 mAbs gebruikt voor de vergelijking van de activiteit van het serum in de TB-test en de competitie-ELISA (hoofdstuk 6) Met deze competitie testen kan 75% van de blokkerende sera uit Kameroen worden geidentificeerd Ook wordt in dit hoofdstuk aannemelijk gemaakt dat als het eiwit afkomstig is van isolaten van malaria parasieten uit verschillende delen van de wereld dit nauwelijks invloed heeft op de resultaten van de daarmee uitgevoerde competitie-ELISA's

In een longitudinale studie met sera van mensen die meer dan 10 jaar in de tropen hebben geleefd en naar Nederland komen met een malaria infectie, blijkt dat de ontwikkeling van TB-activiteit overeenkomt met de ontwikkeling van antistoffen, die een epitoopspecifieke competitie vertonen met Pfs48/45 en in het bijzonder met epitoop 3 (hoofdstuk 7) Uit deze studies blijkt dat de Pfs48/45 competitie-ELISA's al enige voorspellende waarde hebben ten opzichte van de transmissie blokkade activiteit van het geteste serum

Een opmerkelijke waarneming in dit longitudinale onderzoek was dat na blootstelling aan gametocyten de TB-immuniteit in een patient meer dan 80 weken aanhield Voor een vaccin op basis van dit eiwit is het belangrijk te weten hoelang deze humorale immuniteit na vaccinatie aanwezig blijft

De in dit proefschrift beschreven serologische bepalingen die gecorreleerd zijn aan TB-activiteit kunnen mogelijk een bijdrage leveren aan een analyse van de epidemiologie van de transmissie van malaria en zo indirect aan de controle op verspreiding van de ziekte

ABBREVIATIONS

C45-ELISA	anti-Pfs48/45 competition-ELISA
C230-ELISA	anti-Pfs230 competition-ELISA
ELISA	Enzym-Linked Immunosorbent Assays
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FPLC	Fast Protein Liquid Chromatography
HRPO	horseradish peroxidase
IFA	immunofluorescence microscopy with dried parasites as the antigen
Ig	immunoglobulin
kDa	kiloDalton
mAb	monoclonal antibody
NP40	Nonidet P-40
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfs	*Plasmodium falciparum* surface protein
PLL	poly-L-lysine
PMSF	phenyl-methyl-sulphonyl-fluoride
PVDF	polyvinyldifluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIFA	suspension IFA with live parasites as the antigen
TB	transmission-blocking
TX-114	Triton X-114

LIST OF PUBLICATIONS

VERMEULEN AN, WFG ROEFFEN, JC VAN MUNSTER & JHETH MEUWISSEN 1983 Isolation and characterization of membrane proteins of *Plasmodium berghei* sporozoites *Mol Biochem Parasitol* 7:197-207

VERMEULEN AN, ROEFFEN WFG & MEUWISSEN JHETH 1983 *Plasmodium berghei* purification of immunologically active proteins of sporozoites Abstract *Trop Geogr Med* 35:91

VERMEULEN AN, PONNUDURAI T, LENSEN AHW, ROEFFEN WFG & MEUWISSEN JHETH 1983 The purification of *plasmodium falciparum* macrogametes and/or zygotes prepared from in vitro cultures *Trans Roy Soc Trop Med Hyg* 77 753-755

VERMEULEN AN, ROEFFEN WFG HENDERIK JBJ, PONNUDURAI T, BECKERS PJA & MEUWISSEN JHETh 1985 *Plasmodium falciparum* transmission blocking mono-clonal antibodies recognized monovalently expressed epitopes *Develop biol Standard* 62 91-97 (S Krager, Basel 1985)

ROEFFEN W, BECKERS PJA, TEELEN K, VERHAVE JP, ELING W & SAUERWEIN R 1993 Comparison of serological tests and bio-assay for malaria transmission blocking capacity in field sera *Parassitologia* 35 95-97

ROEFFEN W, LENSEN T, MULDER B, TEELEN K, SAUERWFIN R, ELING W, MEUWISSEN JHETH & BECKERS P 1994 Transmission-blocking immunity as observed in a feeder system and serological reactivity to Pfs48/45 and Pfs230 in field sera *Mem Inst Oswaldo Cruz* Rio de Janeiro 89 13-15

ROEFFEN W, BECKERS P, TEELEN K LENSEN T, SAUERWEIN RW, MEUWISSEN JHETH & ELING W 1995 *Plasmodium falciparum* A comparison of the activity of Pfs230-specific antibodies in an assay of transmission-blocking immunity and specific competition ELISAs *Exp Parasitology* 80 15-26

ROEFFEN W, LENSEN T, MULDER B, TEELEN K, SAUERWEIN R, VAN DRUTEN J, ELING W, MEUWISSEN JHETH & BECKERS PA 1995 comparison of transmission-blocking activity with reactivity in a *Plasmodium falciparum* 48/45-kD molecule-specific competition ELISA *Am J Trop Med Hyg* 52 60-65

ROEFFEN W, GEERAEDTS F, ELING W, BECKERS P, LENSEN T & SAUERWEIN R 1995 Transmissi on-blockade of *Plasmodium falciparum* malaria by anti-Pfs230 specific antibodies is isotype dependent *Inf Immun* 63 467-471

MULDER B, ROEFFEN W, SAUERWEIN R, TCHUINKAM T, BOUDIN C & VERHAVE JP 1995 Anti-Pfs25 monoclonal antibody 32F81 blocks transmission from *Plasmodium falciparum* gametocyte carriers in Cameroon 1995 *Trans Roy Soc Trop Med Hyg* 90 195

MOELANS IMD, COHEN J, MARCHAND M, MOLITOR C, DE WILDE P, VAN PELT JF, HOLLINGDALE MR, ROEFFEN WFG, ELING WMC, ATKINSON CT, AIKAWA M, SCHOENMAKERS JGG & KONINGS RNH 1995 Induction of *Plasmodium falciaparum* sporozoite neutralizing antibodies upon vaccination with recombinant Pfs16 vaccina virus and/or recombinant Pfs16 protein produced in yeast *Mol Biochem Parasitol* **72**:179-192

ROEFFEN W, MULDER B, TEELEN K, BOLMER M, ELING W, TARGETT GAT, BECKERS PJA & SAUERWEIN R 1996 Association between anti-Pfs48/45 reactivity and *P falciparum* tranmis sion blocking activity in sera from Cameroon *Parasite Immunol* **18** 103-109

GOUNOUE R, LE GOFF G, MULDER B, ROEFFEN W, VERHAVE JP & BOUDIN C 1996 Anticorps anti-Pfs48/45 dans les serums de deux populations vivant en zone d'endemie palustre de niveaux de transmission differents *Bull Liais doc OCEAC* **29**:16-21

ABSTRACTS AND INTERNATIONAL PRESENTATIONS

Henderik J & Roeffen W 1985 Purification of monoclonal antibodies (moabs) using fast perfor mance liquid chromatography (FPLC) Proceedings First FPLC Symposium

BECKERS P, ROEFFEN W, ARENS T SIEBEN M & MEUWISSEN JHETH 1988 An epitope specific serological assay for the blocking of *Plasmodium falciparum* Abstract XIIth international congres for Tropical Medicine and Malaria Amsterdam

FRIES H, LAMERS M, SIEBEN M, BECKERS P, ROEFFEN W BENSINK T, PONNUDURAI T & MEUWIS SEN JHETH 1988 Biosynthese of a major target antigen (Mr 25 kD) for transmission blocking antibodies of *Plasmodium falciparum* Abstract XIIth international congres for Tropical Medicine and Malaria Amsterdam

ROEFFEN W, BECKERS P, FASSOTTE A, SIEBEN M & MEUWISSEN JHETH 1989 Serological *Plasmodium falciparum* transmission-blocking assay results from endemic malaria regions and travellers BSP Southampton, England

ROEFFEN W, BECKERS PJA, FASSOTTE A, ARENS T, VERHAVE JP & MEUWISSEN JHETH 1990 Antigens expressed on sexual stages of *Plasmodium falciparum* as measured by indirect IFA ICOPA-VII Parijs, Frankrijk

ROEFFEN W, GEERAEDTS F, BECKERS P, TEELEN K, LENSEN T & ELING W 1993 Natural antibo-dy-reactivity to the 230 kilodalton surface protein of the sexual stage of *Plasmodium falciparum* BSP, Oxford, England

ROEFFEN W, TEELEN K, BECKERS P, MULDER L, SAUERWEIN R & ELING W 1994 Development of competition ELISAs for the analysis of transmission-blocking activity in field sera BSP spring meeting, Bath, England

ROEFFEN W, LENSEN T, MULDER B, VAN DRUTEN J, ELING W, BECKERS PJA & SAUERWEIN R 1994 Pfs48/45 protein of *Plasmodium falciparum* as target for a malaria Transmission blocking vaccin Meeting of the Dwgm Vaccines , 19-20-april te Lunteren

ROEFFEN W, BOLMER M, ELING W, LENSEN T & SAUERWEIN R 1994 An IFA for the detection of complement lysis of parasites of the sexual forms of *Plasmodium Falciparum* 6th Malaria Meeting of the BSP, 19-21 september, Liverpool, England Annals of Tropical Medicine and Parasitology (april,95) 89,206

LENSEN A, BOLMER M, VAN GEMERT GJ, ROEFFEN W ELING W & SAUERWEIN R 1994 Activa ted human neutrophils in mosquito bloodmeal reduce transmission of *Plasmodium falciparum* 6th Malaria Meeting of the BSP, 19 21 september 1994, Liverpool, England Annals of Tropical Medicine and Parasitology (april,95) 89,189

ROEFFEN W, GEERAEDTS F, ELING W, LENSEN T & SAUERWEIN R 1994 Blockade of malaria transmission by isotype switch variants NVVI te Noordwijkerhout on 15 and 16 december Nieuwsbrief NVVI, 5/94 page 72

ROEFFEN W, MULDER B, TEELEN K BOLMER M, ELING W, BECKERS PJA & SAUERWEIN R 1995 Anti Pfs48/45 reactivity against gametocytes of *P falciparum* in sera from Cameroon partially predict transmission blocking activity Meeting of the Dwgm VACCINES , april, page 11 12 Lunteren

SAUERWEIN R, ROEFFEN W, MULDER B, LENSEN T, VAN GEMERT GJ, BEETSMA A & ELING W 1995 Transmission-blocking immunity for malaria Meeting of the Dwgm VACCINES april, page 9 Lunteren

ROEFFEN W MULDER B TEELEN K BOLMER M, ELING W, BECKERS PJA & SAUERWEIN R 1995 The mechnisme of antibody mediated transmission blocking activity (Pfs48/45 (5 (sub)-epitopen) reactivity in sera of gametocyte carriers from Cameroon) 3Rd EC Gametocy te network meeting, april 18 19, Nijmegen

ROEFFEN W, TEELEN K JANSSEN J, ELING W, KONINGS R & SAUERWEIN R 1995 The rol of adjuvants in the antibody response against malaria vaccine candidate Pfs48/45 Najaars meeting van de Dwgm VACCINES , november Utrecht

ROEFFEN W TEELEN K BOLMER M, SIEBEN M, ELING W & SAUERWEIN R 1995 The role of antibody isotypes on sexual stage immunity of *P falciparum* malaria BSI/NVVI, 6 8 december, Brighton, England Immunology 86 suppl 1 135

ROEFFEN W, TEELEN K VAN DE VEGTE-BOLMER M, ELING W & SAUERWEIN R 1996 Antibody and transmission blocking activity of human serum after a *P falciparum* infection 8th Malari a Meeting of the BSP 9-11 september 1996, Glasgow England

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 11 Oktober 1950 te Maasniel In 1971 behaalde hij het diploma HBO-a aan de Analistenschool te Venlo Na 4 jaar te hebben gewerkt bij Diosynth te Oss begon hij in 1976 met zijn werkzaamheden in het Academisch Ziekenhuis 'St Radboud' te Nijmegen De eerste 5 jaar was hij werkzaam bij Dr J H M van Tongeren op het laboratorium van Maag- Darm en Leverziekten Sinds 1981 is hij betrokken bij het biochemisch en immunologisch onderzoek op het gebied van de transmissie van malaria en de ontwikkeling van een transmissie blokkerend vaccin op het laboratorium van de Medische Microbiologie, sectie Parasitologie Na het behalen van zijn HLO diploma Biologie aan de Hoge school van Amsterdam in 1992 begon hij met het wetenschappelijk onderzoek dat beschreven staat in dit proefschrift, aanvankelijk onder leiding van Prof Dr J H E Th Meuwissen en na diens emeritaat onder leiding van Dr R W Sauerwein en Dr W M C Eling

WOORDEN VAN DANK

De schrijver van een proefschrift zou men kunnen vergelijken met een waterpoloer, die weliswaar veel individueel werk moet verzetten, maar slechts succesvol kan zijn door met de andere teamleden samen te werken Bij deze wil ik dan ook allen die aan het tot stand komen van dit proefschrift hebben meegewerkt van harte bedanken

Zoals elk team een trainer en een coach kent, bedank ik mijn "trainers en coaches" bij deze Als eerste wil ik mijn promotor Joep Meuwissen noemen voor de kans die hij mij gaf een begin te maken met het wetenschappelijk onderzoek Na zijn emeritaat werd de begeleiding overgenomen door mijn copromotoren Wijnand Eling en Robert Sauerwein Robert Sauerwein stelde mij in staat veel meer facetten van het malaria onderzoek te leren kennen met Wijnand Eling en Pieter Beckers heb ik vele uren van wetenschappelijke discussies doorgebracht Allen hebben mij intensief begeleid bij de vele werkzaamheden en mijn immunologisch blikveld daarmee sterk verbreed

Elk team bestaat uit een aantal spelers die op hun manier een eigen bijdrage leveren om tot scoren te komen Karina Teelen die vanaf het begin tot het gereedkomen van dit proefschrift actief betrokken was bij vrijwel alle experimenten, bedank ik voor haar inspiratie, accuratesse, doorzettings-vermogen en vriendschap Verder bedank ik Marianne Sieben en Truus Derks voor de bereiding van muis en rat monoclonalen, Marga Bolmer, Arianne Huisman, Geert-Jan van Gemert, Jo Hooghof en Jolanda Remmers voor de kweek van vele parasieten en de transmissie blokkade experimenten, Theo van de Ing, Geert Poelen en Jan Koedam van het Centraal Dieren laboratorium voor hulp bij de dierproeven Tevens bedank ik alle AIO en AIO-achtigen, met wie ik mijn kamer gedeeld heb, voor hun opbouwende en bemoedigende kritiek waarbij ik de namen van Annette Beetsma, Roos Perenboom, Rob Hermsen en Ton Lensen niet onvermeld wil laten

Buiten de teamleden zijn er nog diverse mensen op de achtergrond die er indirect voor zorgen dat het team niets te kort komt De mensen van het lab die indirect hun bijdrage hebben geleverd zoals Jan Peter Verhave, Theo Arens en Tita Oetinger dank ik hierbij, Bert Mulder en de medewerkers van het OCEAC/ORSTOM voor het verzamelen van de Kameroenese sera en voor de begeleiding tijdens mijn eerste tropenervaring

Als een speler al meerdere jaren voor een team speelt kent hij ook oud-teamleden Alle (ex)-medewerkers van de sectie Parasitologie dank ik voor hun getoonde bereidwilligheid tot samenwerking In het bijzonder wil ik hier noemen Felix Geeraedts die in het kader van zijn hoofdvak aan dit onderzoek heeft bijgedragen

Het is onmogelijk om alle mensen die hebben bijgedragen aan dit onderzoek afzonderlijk te bedanken Ik heb vooral de laatste jaren geleerd dat goed onderzoek alleen mogelijk is binnen een kritische afdeling waar een goede sfeer heerst

De publikatie van dit proefschrift kwam mede tot stand dankzij de financiële steun van;

- Roche Nederland B.V.
- Nederlandse Vereniging van bioMedisch Laboratoriummedewerkers (NVML)
- Bio-Rad Laboratories B.V.

Stellingen

behorende bij het proefschrift van Will Roeffen

Plasmodium falciparum: Relevance of human antibodies for blocking transmission of the parasite from man to mosquito.

Nijmegen, 12 november 1996.

De goede correlatie tussen de activiteit in de anti-Pfs48/45 specifieke competitie ELISA's en de transmissie-blokkade bioassay maakt de serologische testen geschikt voor onderzoek naar de epidemiologische betekenis van transmissie-blokkerende immuniteit. (dit proefschrift)

Gametocytaemie bij een patiënt met een *P.falciparum* infectie is niet maatgevend voor de transmissie-blokkerende activiteit in het serum. (dit proefschrift)

Het aantal voorafgaande *P.falciparum* infecties waarbij gametocyten tot ontwikkeling zijn gekomen bepalen de transmissie-blokkerende activiteit van het serum en de specifieke serologische reactiviteit. (dit proefschrift)

De complement afhankelijke anti-Pfs230 antilichaam gemediëerde transmissie-blokkade die met muis monoclonalen antistoffen wordt waargenomen is vooralsnog niet bij de mens aantoonbaar. (dit proefschrift)

Hoewel een transmissie-blokkade vaccin geen individuele bescherming biedt tegen malaria komt het effect van zo'n vaccinatie overeen met de vaccinatie van jongetjes met het Rubella-vaccin die bijdraagt aan de opbouw van de herd-immunity.

Worminfecties kunnen de immunologische responsiviteit polariseren en zelfs verlammen. Het zou daarom verstandig zijn om in "het veld" de te vaccineren personen (malaria-vaccin) eerst te behandelen met anthelmintica. (Clin Exp Immunol (1996)**103**:239-243, Ind J Med Res (1996)**103**:46-54.

Het is de vraag of bij de ontwikkeling van een vaccin het gaat om de keuze van het juiste epitoop of om de toevoeging van het juiste adjuvants.

Om de efficiëntie van natuurwetenschappelijk onderzoek te vergroten moet de rapportage van negatief uitgevallen onderzoeksresultaten, die voor de voortgang van het onderzoek belangrijk zijn, worden bevorderd.

Door de commotie die ontstaan is rond de met BSE besmette Britse koeien is men dodelijk benauwd geworden voor de ziekte van Creutzfeldt-Jacob. In analogie met het gevoerde beleid zou nu ook de verkoop van GSM-telefoons verboden moeten worden in verband met het risico van de ontwikkeling van hersentumoren.

De tijdwinst die verkregen werd door de introductie van computer-programma's zoals tekstverwerkers, statistische paketten, spreadsheets, databases etc., wordt onderschat door de tijd die besteed moet worden om de (on)mogelijkheden van deze programma's te leren gebruiken.

De hedendaagse beschikbaarheid van communicatiemiddelen zoals Fax, Email, Internet, etc., vergroot niet alleen het aantal contacten, maar ook het aantal misverstanden.

Een goede biomedische onderzoeker is een goede analist.