Inhibitory monoclonal antibodies to soluble *Plasmodium falciparum* antigens

P.H. Jakobsen[1], S. Jepsen[1], and R. Agger[2]

[1] Malaria Research Laboratory, Treponematoses Department, Statens Seruminstitut, Copenhagen, Denmark
[2] The Central Hybridoma Laboratory, Statens Seruminstitut, Copenhagen, Denmark

Abstract. Several murine monoclonal antibodies were raised against purified soluble *Plasmodium falciparum* antigens from the asexual blood stage. The monoclonal antibodies were purified from ascites by preparative agarose gel electrophoresis and tested for inhibitory activities against *P. falciparum* in vitro cultures. One monoclonal antibody, HATR 2–4, showed an isolate-specific growth inhibition of *P. falciparum* in vitro cultures. The antibody reacted in immunoblotting with bands of 250 and 57 kilo dalton (kdalton). Another monoclonal antibody, HATR 2–8, showed growth inhibition of several geographically distinct *P. falciparum* isolates. HATR 2–8 reacted in immunoblotting with bands of 250 and 74 kdalton. Heating of the antigens destroyed the reactivity of HATR 2–8. The monoclonal antibodies HATR 2–4 and HATR 2–8 probably recognize different epitopes on the same antigen. This antigen circulates in the plasma of some patients with *P. falciparum* parasitaemia.

Soluble antigens of *Plasmodium falciparum* are present in plasma of patients with *P. falciparum* malaria (Wilson et al. 1969; Jepsen and Axelsen 1980) and in the supernatant of *P. falciparum* in vitro cultures (Wilson et al. 1973; Jepsen and Andersen 1981).

The soluble antigens constitute a heterogeneous group of proteins divided into labile, resistant and stable (S) antigens on the basis of their temperature susceptibility (Wilson et al. 1973). S antigens show considerable serological diversity (McGregor and Wilson 1971) and they constitute a stable antigen group as *P. falciparum* isolates

retain their S-antigenic phenotype over many passages in vitro and in vivo (Wilson 1980). The soluble antigens are probably liberated to the plasma late in the schizogony when the erythrocyte ruptures and during merozoite invasion. Soluble antigens from patients and the soluble antigens from in vitro cultures are immunochemically identical (Jepsen and Andersen 1981).

Soluble antigens may play a role as inducers of protective immunity to *P. falciparum* malaria since population studies in the Gambia have pointed to a correlation between clinical immunity and the possession of precipitating antibodies to soluble *P. falciparum* antigens (McGregor and Wilson 1971). Furthermore, affinity-purified human IgG antibodies to soluble antigens inhibit *P. falciparum* in vitro cultures (Jepsen 1983). Thus soluble antigens may well be candidates for a malaria vaccine. We have therefore raised murine monoclonal antibodies against affinity-purified soluble *P. falciparum* antigens from in vitro cultures with the aim of isolating and characterizing the corresponding antigens. We here report the production and characterization of two monoclonal antibodies inhibitory to *P. falciparum* growth in vitro. The corresponding antigen has been isolated by affinity chromatography and shown to correspond to an antigen naturally circulating in the plasma of *P. falciparum*-infected individuals.

Materials and methods

Parasite cultures

Plasmodium falciparum isolates, F32/Tanzania, L1/Liberia, FCD41/India and FCD28/Senegal were kept in continuous culture in a modified Trager and Jensen system (Trager and Jensen 1976) at 37°C with a gas mixture of 10% O_2, 5% CO_2 and 85% N_2. The medium, RPMI 1640 supplemented with 21 mM sodium bicarbonate, 25 mM HEPES buffer and 10% human serum, was changed daily. The parasites were grown in erythro-

cytes, blood group A, 10% v/v. For biosynthetic labelling of the parasite ^{35}S-methionine, 40 µCi per ml culture was used for 24 h in methionine-deficient RPMI 1640.

Sera

Sera from Liberian healthy adult blood donors clinically immune to malaria were screened for precipitating antibodies to soluble antigens by crossed immunoelectrophoresis (CIE) (Jepsen and Axelsen 1980). Immunoadsorbent isolated *P. falciparum* antigens from supernatant of continuous in vitro cultures (Jepsen and Andersen 1981) were used as antigens. Ten sera with high titres of precipitating antibodies were pooled and used as immune reference sera pool (ISP).

Isolation of antigens

The affinity purification of soluble *P. falciparum* antigens has previously been reported in detail (Jepsen and Andersen 1981). A parasite sonicate used as antigen for the enzyme-linked immunosorbent assay (ELISA) and for immunoblotting was produced as follows: at 20–30% parasitaemia, the erythrocytes were lysed by adding guinea-pig complement and rabbit anti-human erythrocyte membrane antibody (Dakopatts, Denmark). The lysed suspension was centrifuged for 5 min at 400 g. The supernatant containing liberated parasites was conserved. The sediment was washed 4 times with saline. The resulting five supernatants were pooled and centrifuged for 30 min at 17000 g. The supernatant was discarded and the pellet resuspended in saline and sonicated 3 × 30 s with 10 s intervals on an ice bath.

Isolation of single antigens

This involved 11.5 ml murine monoclonal IgG purified by agarose gel electrophoresis (2 mg/ml) being coupled to 50 ml divinyl sulfone agarose according to the manufacturers instruction (KemEnTec, Denmark). Then 350 ml supernatants from the daily medium refreshment of the cultures were pooled, filtered through a 0.2 µm Millipore filter and, after dialysis, added to the column. After washing with column buffer, bound antigen was eluted with 3 M KSCN.

Immunization

BALB/cJ mice (♀) 3- to 6-months-old were immunized with affinity-purified soluble antigens. Antigen mixed 1:1 with Freund's complete adjuvans was injected subcutaneously 3 or 4 times with 2-week-intervals. Antigen without adjuvant was given intravenously or intraperitoneally 3 days before the isolation of spleen cells.

Hybridomas

Spleen cells from the immunized mice were fused with X 63 Ag8-653 myelomacells. Fusion was carried out essentially as described by Reading (1982). Hybridoma cell cultures were grown in Dulbecco's MEM plus 10% foetal calf serum. Hybridoma cultures producing antiplasmodial antibodies were identified (see later) and selected hybrids were cloned by limiting dilution on a feeder layer of peritoneal cells from BALB/cJ mice. Ascites was produced by injecting 5×10^6–10^7 hybridoma cells intraperitoneally in irradiated (300 RAD) BALB/cJ mice. Monoclonal antibody isotype was determined by gel precipitation (Ouchterlony) or by an ELISA test with rabbit anti-mouse immunoglobulin sublcass antibodies (Dakopatts, Denmark).

ELISA

Hybridoma culture supernatants were screened for content of antibodies reacting with (1) whole parasite sonicate and (2) human serum. Supernatants from selected monoclonal hybrid cell lines with high titres in the antiplasmodial ELISA were also screened for reactivity against a sonicate of human erythrocytes. The coating antigen in this ELISA were non-infected A positive erythrocytes from the same batch used for parasite cultures. Procedures for coating, washing, incubation etc. are reported in detail elsewhere (Schapira et al. 1984). The conjugate was a rabbit anti-mouse immunoglobuline conjugated with peroxidase (Dakopatts, Denmark).

In vitro inhibition

Monoclonal antibodies were purified from ascites by preparative agarose gel electrophoresis (Carlström and Johansson 1983). The gel fraction containing the monoclonal antibody was cut out and centrifuged at 15000 g for 15 min. The supernatant containing the monoclonal antibody was tested for reactivity against *P. falciparum* antigens by ELISA and the purity was confirmed by agarose gel electrophoresis. The purified antibody was filtered through a 0.22 µm Millipore filter, dialysed against culture medium for 48 h and the protein content estimated by a dye binding assay (Bio Rad). Monoclonal antibodies were tested for inhibitory activity against asynchronous *P. falciparum* isolates by a modified Desjardins test (Desjardins et al. 1979).

For each monoclonal antibody, 3 horizontal rows of 7 serial 2-fold dilutions in 50 µl RPMI 1640 were prepared on a 96-well Nunclon (Nunc, Denmark) microculture plate, with two control wells with infected erythrocytes and one blank well with unparasitised RBC in each row. Thereafter, 50 µl of parasitised erythrocytes with 1%–1.7% parasitaemia was placed in each well except the blank one. Final hematocrit was 1.5%. The ^{3}H-hypoxanthine (Nuclear, New England) 0.4 µCi in 20 µl complete medium was added to each well after 48 h. The plates were agitated and incubated for additional 18–20 h. The parasites were harvested by a Skantron (Norway) Automatic Cell Harvester onto glass fibre filters (Titertek), washed for 30 s with distilled water and then for 60 s with 0.9% NaCl. The harvested material was counted in toluene scintillation fluid (Aqualuma Plus) for 2 min in an Isocap/300 beta liquid scintillation counter. Activity bound was calculated as $P = ((cpm/sample) - cpm (blank))/(Cpm(control) - cpm (blank))$, where cpm(sample) was the mean of the three values corresponding to one monoclonal antibody concentration, cpm(blank) was the mean of all blank values and cpm(control) was the mean of all control values from one plate. Percent inhibition of the parasite culture is drawn as a function of the concentration (µg Ig/ml culture) of purified monoclonal antibodies.

Crossed immunoelectrophoresis (CIE)

This involved 20 µl of the affinity-purified antigenpreparation or 40 µl of ^{35}S-methionine-labelled crude culture supernatant (Jepsen 1983) plus 40 µl of the affinity-purified antigen preparation being run in the first dimension gel at 10–15 V cm^{-1} for 30 min. The second dimensional gel was run perpendicular to the first dimension at 2 V cm^{-1} overnight into a gel containing 400 µl ISP. The plates were washed, pressed and stained with Coomassie brilliant blue. In a series of experiments, the antigen was heated at 100° C for 5 min before the electrophoresis. The plates with ^{35}S-methionine-labelled material were incubated with a LKB Ultrofilm (LKB, Sweden) at −80° C for approximately four weeks. Tandem crossed immunoelectrophoresis was performed as described by Jepsen and Andersen (1981).

Immunoblotting

Here, 400 µl supernatant of the parasite sonicate (2.2 mg protein/ml) was used as antigen for immunoblotting. The antigens

were applied on a SDS-PAGE slab gel consisting of 8% stacking gel and 10%–20% discontinuous separation gel. Electrophoresis was performed at 25–40 V for 17 h under reducing and non-reducing conditions and with or without prior heating of the antigens at 100° C. The antigens were then electrotransferred to nitrocellulose sheets in a Bio Rad Trans Blot cell. The nitrocellulose strips (55 µg protein/strip) were blocked with PBS + 0.5% BSA + 0.5% Tween 20 and incubated with diluted monoclonal antibodies (m.ab's) and washed. The sheets were stained after incubation with peroxidase conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Denmark). Molecular weight standards were stained with amidoblack.

Results

Crossed immuno electrophoresis

The crossed immuno electrophoresis (CIE) of affinity-purified soluble *P. falciparum* antigens against ISP resulted in more than ten different precipitates. Heating of the antigen solution to 100° C for 5 min prior to the electrophoresis showed that most of the antigens could be classified as heat stable antigens. ^{35}S-methionine biosynthetic labelling of the parasite culture followed by CIE of the soluble antigens and autoradiography confirmed that the antigens precipitated by ISP were synthesized by the parasites.

Hybridomas

From three fusions, several monoclonal antibodies were produced. All the selected monoclonal antibodies were reactive against parasite antigens and non-reactive against human serumproteins in EL-ISA. Table 1 shows the Ig subclass of three monoclonal antibodies and the type of antigen used for immunization.

Inhibition assays

Fig. 1 shows the results of the in vitro parasite multiplication inhibition tests for three monoclonal antibodies against the F32 and FCD41 *P. falciparum* isolates. All tests were carried out with asynchronous cultures. M.ab. HATR 2–7 showed no significant inhibitory effect on the parasite multiplication. M.ab. HATR 2–4 showed a 50% inhibition of the F32 parasite growth at a concentration of 160 µg IgG/ml culture and a 95% inhibition at a concentration of 470 µg IgG/ml culture. M.ab. HATR 2–8 had a 50% inhibition at a concentration of 51 µg IgG/ml culture and a 95% inhibition at a concentration of 135 µg IgG/ml culture. M.ab. HATR 2–8 inhibited the parasite growth of *P. falciparum* isolates FCD41/Thailand and FCD 28/Senegal (data not shown) at similar concentrations to those for the F32 *P. falciparum* isolate, while

Table 1. Characteristics of three monoclonal antibodies reactive with *Plasmodium falciparum* antigens

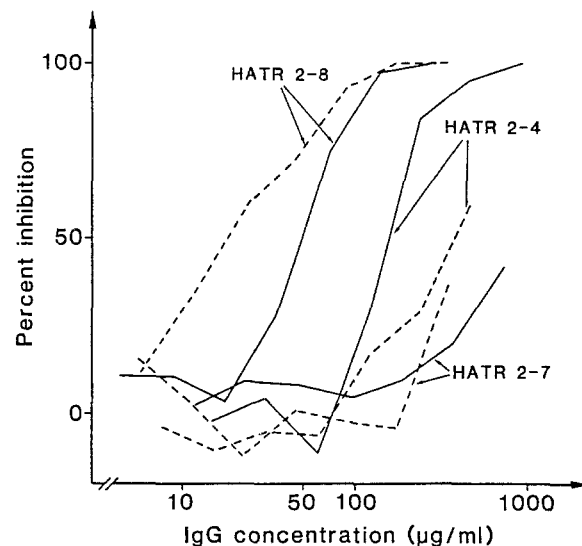| Monoclonal antibody	Isotype	Derived from mice immunised with
HATR 2–4	IgG$_1$, k	Soluble antigens from F32 isolate
HATR 2–7	IgG$_{2a}$, k	Soluble antigens from F32 isolate
HATR 2–8	IgG$_1$, k	Soluble antigens from L 1 isolate

Fig. 1. Inhibition of growth of *Plasmodium falciparum* isolates F32 and FCD41 in vitro by purified murine monoclonal antibodies. —— results parasite isolate F32; – – – – results with parasite isolate FCD41

m.ab. HATR 2–4 and m.ab. HATR 2–7 gave no significant inhibition of the FCD 41 and the FCD28 isolates.

Immunoblotting

Figure 2 shows the reactivity of the m.ab.'s HATR 2–4, 2–7 and 2–8 with a parasite sonicate. When the electrophoresis of the antigen solution was performed after heating and reduction, m.ab.'s HATR 2–4 (Fig. 2b/I) and HATR 2–8 (Fig. 2c/I) did not show any reactivity while m.ab. HATR 2–7 reacted with polypeptide bands of app. 31 kdalton and 10–16 kdalton (Fig. 2a/II). When the antigen solution was not heated, m.ab. HATR 2–4 reacted with 250 and 57 kdalton antigens under reducing conditions (Fig. 2b/II) and with a 250 kdalton antigen under non-reducing conditions (Fig. 2b/III). M.ab. HATR 2–8 reacted with an ill-defined broad band in the range of 250 kdalton under reducing
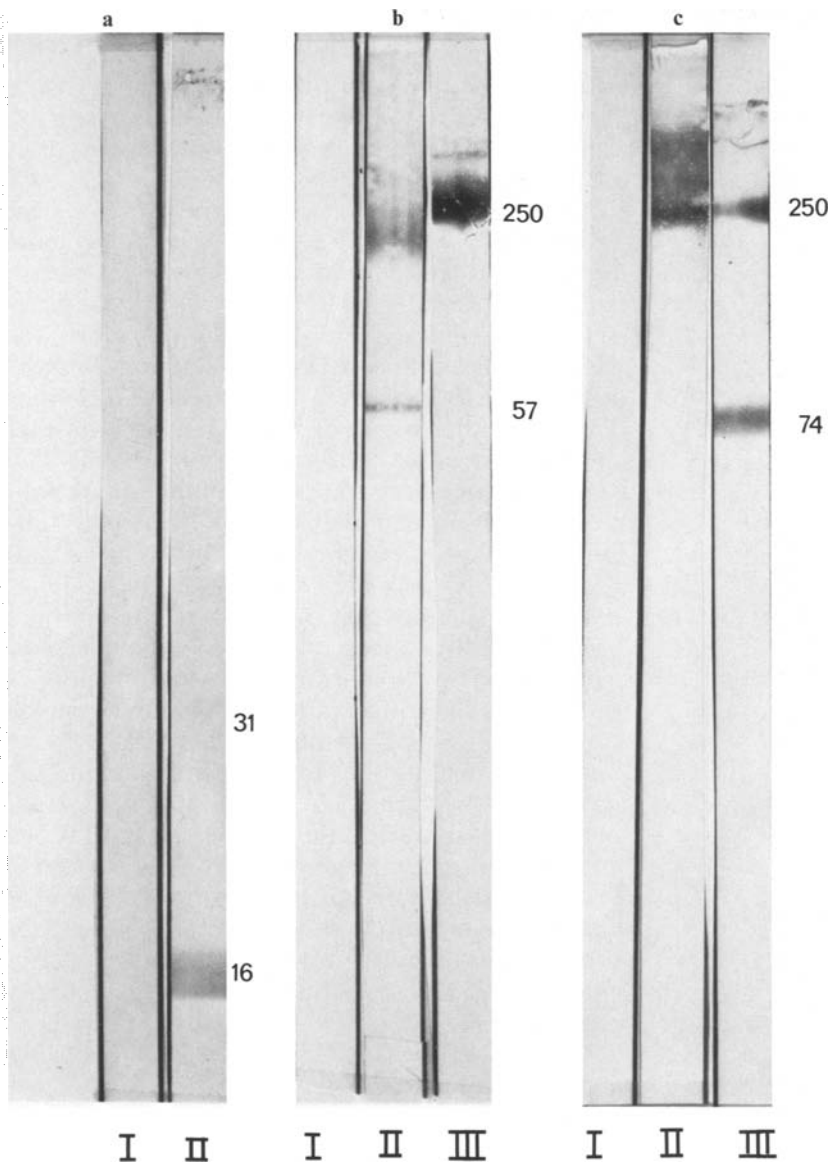
Fig. 2a–c. Reactivity of monoclonal antibodies against a parasite sonicate (F32 isolate) in immunoblotting. Marker proteins were: Myosin (200 kdalton), Beta-Galactosidase (116.25 kdalton), Phosphorylase b (92.6 kdalton), Bovine Serum Albumin (66.2 kdalton), Ovalbumin (45 kdalton), Carbonic Anhydrase (31 kdalton, Soybean Trypsin Inhibitor (21.5 kdalton) and Lysozyme (14.4 kdalton). **a** The buffer showed no reactivity (*I*) regardless of the electrophoresis condition. M.ab. HATR 2–7 diluted 1:50 showed similar reactivity with and without heating the antigens under reducing and non-reducing conditions (*II*). **b** Reactivity of m.ab. HATR 2–4 diluted 1:10 (*I*) under reducing conditions and with prior heating of the antigens, (*II*) Under reducing conditions but without heating the antigens and (*III*) under non-reducing conditions and without heating the antigens. **c** Reactivity of m.ab. HATR 2–8 diluted 1:10 (*I*) under reducing conditions and with prior heating of the antigens, (II) under reducing conditions but without heating the antigens and (III) under non-reducing conditions and without heating the antigens

conditions (Fig. 2c/II), and with a 250 kdalton and an approximately 74 kdalton antigen under non-reducing conditions (Fig. 2c/III). The reactivity of m.ab. HATR 2–7 did not change.

Isolation of single antigens

The antigen eluted from the columns using as ligand either m.ab. HATR 2–4 or HATR 2–8 was tested in CIE and tandem CIE. The eluted antigen showed an electrophoretic pattern similar to that of antigen no. 1 (Jepsen and Axelsen 1980). Figure 3a, b, c show the complete fusion with precipitate no. 1 when the eluted antigen was run together with plasma from an acutely ill malaria patient. The eluted antigen reacted with Liberian, Gambian and Indonesian immune sera.

Discussion

Reports have indicated that soluble antigens from *P. falciparum* in vitro cultures may induce protective immunity and consequently might be candidates for a malaria vaccine. Soluble antigens from *P. falciparum* in vitro cultures partly purified by cation-exchange chromatography induced a significant clinical protection in squirrel monkeys against *P. falciparum* infection (James et al. 1985). Soluble antigens of 155 and 130 kdalton, originating from the merozoite surface and isolated from the culture supernatant, bind to glycophorin (Perkins 1984), which may constitute at least part of the erythrocyte receptor for *P. falciparum* merozoites (Perkins 1984). Rabbit antibodies directed against the two antigens inhibit *P. falciparum* in
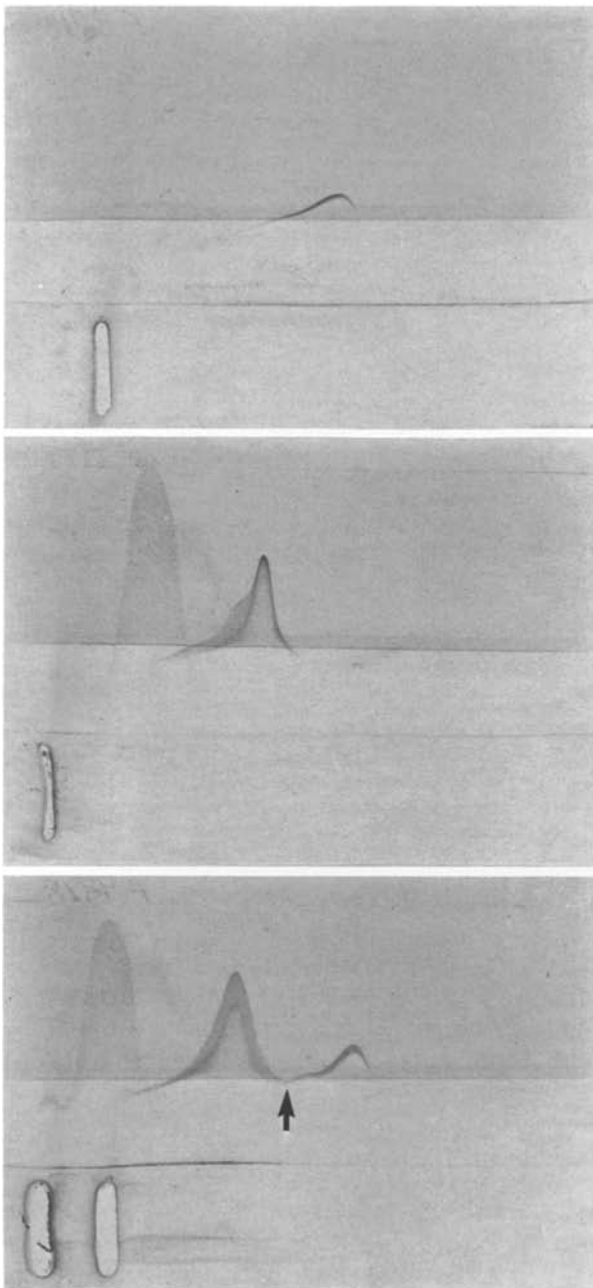
Fig. 3a–c. Tandem crossed immunoelectrophoresis of antigens from the plasma of a *P. falciparum* infected individual and the affinity purified antigen no. 1. Electrophoresis was performed with 30 µl antigen no. 1 isolated by affinity chromatography with either 20 µl plasma from the infected individual or with 20 or 30 µl NaCl in the first dimension at 10–15 V/cm for 30 min. The second dimension was run perpendicular to the first at 2V/cm for 18 h (anode at the top). The second dimension gel contained 300 µl ISP. **a** With NaCl and the isolated antigen no. 1; **b** with plasma from the infected individual and NaCl; **c** with plasma from the infected individual and the isolated antigen no. 1. *Arrow* points to the fusion of the precipitates

vitro (Perkins 1984). Murine monoclonal antibodies against 140, 82 and 41 kdalton antigens inhibit *P. falciparum* in vitro (Perrin et al. 1981). These antigens are present in the culture supernatant in greater amounts than in infected erythrocytes (Rodriques et al. 1983).

As a part of a malaria vaccine development programme, we raised several murine monoclonal antibodies against purified soluble *P. falciparum* antigens in an attempt to isolate and further characterize possible vaccine candidates. Two of the monoclonal antibodies efficiently inhibit *P. falciparum* growth in vitro. The m.ab. HATR 2–4 inhibits only the growth of isolate F32 and not that of other isolates. Although m.ab. HATR 2–4 shows an isolate-specific growth inhibition, the antibody reacts in immunoblotting with soluble antigen from several geographically different isolates (data not shown). This seems controversial but could be explained if the corresponding epitopes are differently exposed from parasite isolate to isolate or if both constant and variant epitopes take part in the antigen-antibody immune complex formation. M.ab.'s probably have a varying number of binding sites to corresponding antigens as reported by Ghosh and Campbell (1986). If all binding sites in the reaction with m.ab. HATR 2–8 are constant epitopes but those for m.ab. HATR 2–4 are located both on the constant and variable epitopes, both m.ab.'s would detect the same peptide band in immunoblotting but elicit the differences in in vitro inhibition of the parasite cultures reported here.

The epitope detected by HATR 2–4 is probably a conformation epitope since the reactivity with m.ab. HATR 2–4 is lost when the antigen solution used for SDS-PAGE is processed after prior heating under reducing conditions. In immunoblotting analysis, HATR 2–4 detects antigens of approximately 250 and 57 kdalton. The treatment of the antigen solution determines which of the bands is detected. m.ab.

HATR 2–8 at very low concentrations inhibits the in vitro growth of all *P. falciparum* isolates tested so far, including the chloroquine sensitive isolate F32 from Tanzania, the chloroquine and Fandidar® resistant isolate FCD41 from India and the pyrimethamine and cycloguanil resistant but chloroquine sensitive FCD28 isolate from Senegal. Soluble antigens from the Liberian *P. falciparum* isolate L1 were used immunization. It seems likely that the HATR 2–8 antibody is directed against a possibly protective antigen shared by geographically separated isolates. HATR 2–8 reacts with 250 and 74 kdalton antigens in immunoblotting, prob-

ably recognising a conformation epitope, as HATR 2–4, since heating of the antigen solution destroys the reactivity. Reduction of the antigen solution diminishes the reactivity of HATR 2–8 with the 250 kdalton antigen, supporting the hypothesis that HATR 2–8 recognizes a conformation epitope.

Affinity purification of the m.ab.'s corresponding antigens shows that the antibodies react with epitopes on antigen no. 1. The isolated antigen is immunochemically identical to the antigen most frequently found in plasma of *P. falciparum*-infected Liberian children. The antigen has also been found in most Danish patients suffering from acute *P. falciparum* malaria.

Antigen no. 1, an amphifilic glycoprotein, may during the course of malaria act as a weak immunogene, and protective antibody against this antigen may be formed only gradually. However, when immunizing mice with affinity-purified antigen solutions containing more than ten different antigens, antigen no. 1. acts as a strong immunogen: most monoclonal antibodies obtained reacted with it. Thus antigen no. 1. seems to be a good candidate for a malariavaccine.

References

Carlström A, Johansson BG (1983) Agarose gel electrophoresis – immunofixation. Scand J Immunol [Suppl] 10:23–32

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD (1979) Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agent Chemother 16:710–718

Ghosh S, Campbell AM (1986) Multispecific monoclonal antibodies. Immun Today 7:217–22

James MA, Kakoma J, Ristic M, Cagnard M (1985) Induction of protective immunity to *Plasmodium falciparum* in Saimiri sciureus monkeys with partially purified exoantigens. Infect Immun 49:476–480

Jepsen S (1983) Inhibition of in vitro growth of *Plasmodium falciparum* by purified antimalarial human IgG antibodies. Scand J Immunol 18:567–571

Jepsen S, Andersen BJ (1981) Immunosorbent isolation of antigens from the culture medium of in vitro cultivated *Plasmodium falciparum*. Acta Pathol Microbiol Immunol Scand [C] 89:99–103

Jepsen S, Axelsen NH (1980) Antigens and antibodies in *Plasmodium falciparum* malaria studied by immunoelectrophoretic methods. Acta Pathol Microbiol Immunol Scand [C] 88:263–270

McGregor IA, Wilson RJM (1971) Precipitating antibodies and immunoglobulins in *P. falciparum* infections in the Gambia, West Africa. Trans R Soc Trop Med Hyg 65:135–151

Perkins ME (1984) Surface proteins of *Plasmodium falciparum* merozoites binding to the erythrocyte receptor, glycophorin. J Exp Med 160:788–798

Perrin LH, Ramirez E, Lambert PH, Miescher PA (1981) Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies. Nature 289:301–303

Reading CL (1982) Theory and methods for immunization in culture and monoclonal antibody production. J Immunol Meth 53:261–291

Rodriquez Da Silva L, Loche M, Dayal R, Perrin LH (1983) *Plasmodium falciparum* polypeptides released during in vitro cultivation. Bull WHO 61:105–112

Schapira A, Fogh S, Jepsen S, Pedersen NS (1984) Detection of antibodies to malaria: comparison of results with ELISA, IFAT and crossed immunoelectrophoresis. Acta Pathol Microbiol Immunol Scand [B] 92:299–304

Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. Science 193:673–675

Wilson RJM (1980) Serotyping *Plasmodium falciparum* malaria with S-antigens. Nature 284:451–452

Wilson RJM, McGregor IA, Hall P, Williams K, Bartholomew R (1969) Antigens associated with *Plasmodium falciparum* infections in man. Lancet II:201–205

Wilson RJM, McGregor IA, Wilson ME (1973) The stability and fractionation of malarial antigens from the blood of Africans infected with *Plasmodium falciparum*. Int J Parasitol 3:511–520