DETECTION OF CIRCULATING PLASMODIAL ANTIGENS IN HUMAN SERA BY SANDWICH ELISA WITH MONOCLONAL ANTIBODIES

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Abstract. Two monoclonal antibodies (MAbs), one produced against *Plasmodium falciparum* (PF-IG8) and the other against *P. cynomolgi* (PC-IE12) schizont antigens were used in a sandwich ELISA for the detection of circulating plasmodial antigens in sera of patients infected with either *P. falciparum, P. vivax* or *P. malariae.* The mean ± SD optical density (OD) values for the normal control group using PF-108 and PC-1E12 were 0.351 ± 0.036 and 0.205 ± 0.044, respectively. Mean OD values for the three infected groups were found to be significantly higher than those of the normal control group for both MAbs. However, ELISA values for individual serum specimens did not correlate with the level of parasitemia in the infected blood. Using a cut-off point of mean OD ± 3 SD of the normal control group as indicating a positive reading, the specificity of this assay with both MAbs was 100%. The sensitivity of the assay using PF-1G8 was 95% while that obtained with PC-1E12 was 98%.

INTRODUCTION

The diagnosis of a current malaria infection through the demonstration of parasites in a stained blood film is sensitive and specific (Bruce-Chwatt, 1984) but has the disadvantage of being time-consuming and requires skilled personnel. Alternative methods such as serological assays could make malaria diagnosis more repid. However, the presence of specific antibodies detected through the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) (Spencer *et al,* 1981) only reflects exposure to the parasite and cannot differentiate between current and past infections. Furthermore, several factors such as age, duration and intensity of infection and previous exposure affect the level of antibody response. Serological methods for the detection of plasmodial antigens present in infected blood during current or a recent past infection would therefore be valuable in individual diagnosis, in epidemiological studies and also for the evaluation of malaria vaccines. Such antigens include soluble antigens present in the patient's serum during or shortly after a malaria infection (McGregor *et al,* 1968; Wilson *et al,* 1969) or integral antigens of the parasite present within infected erythrocytes (Bidwell and Voller, 1981; Mackey *et al,* 1980a).

A number of tests has been employed to detect plasmodial antigens in sera of infected humans and murines. A radioimmunoassay (RIA) was developed to detect bloodstage parasites in *Plasmodium berghei* infected mice (Mackey *et al,* 1980b) as well as in *Plasmodium falciparum* infected humans (Mackey *et al,* 1982). Competition ELISA and inhibition ELISA methods have also been used to detect experimental *P. falciparum* infections in monkeys but both these tests appeared to be less sensitive than the RIA (Bidwell and Voller, 1981). Soluble malaria antigens were detected in the sera of *P. falciparum* infected patients using gel precipitation and counter-current immunoelectrophoresis, but these tests showed low sensitivity (McGregor and Wilson, 1971; Perrin *et al,* 1979).

Monoclonal antibodies (MAbs) with defined specificities could theoretically be used as immunodiagnostic reagents for the detection of plassmodial antigens. Such MAbs have been produced and evaluated for their usefulness in plasmodial antigen detection in sera of malaria patients (Soulier *et al,* 1985) and mice infected with *P. yoelii (Taylor et al,* 1986).

In the present study, MAbs produced against *P. falciparum* and *P. cynomolgi* schizont antigens

were evaluated in a sandwich ELISA to determine their usefulness for plasmodial antigen detection in the sera of patients with *P. falciparum, P. vivax* and *P. malariae* infections.

MATERIALS AND METHODS

Malaria patients' sera

Serum samples were collected from malaria patients from the General Hospital, Kuala Lumpur and Gombak Hospital, Selangor Darul Ehsan. A total of 64 sera were used in this study. Of these, 30 were from patients infected with *P. falciparum,* 21 with *P. vivax* and 13 with *P. malariae.* Sera were separated from venous blood samples and stored at -20°C until used.

The presence of malaria parasites in these serum donors was determined by microscopic examination of thick and thin blood films stained with Giemsa. The parasitemias (number of asexual/sexual parasites per µl blood) of the three groups of patients showed a wide range of values, this being 32-32,840, 40-22,240 and 32-2,400 parasites per µl blood in *P. falciparum, P. vivax* and *P. malariae* infections, respectively. The control group of sera was obtained from 50 normal, uninfected persons living in non-endemic malaria areas.

Rabbit polyvalent antisera

Antisera against the schizont antigens of *P. falciparum* (Gombak C) and *P. cynomolgi* (Strain 1) were produced in laboratory-bred rabbits. Rabbits were immunized with six doses of antigen subcutaneously followed by an intravenous booster of soluble antigen consisting of 0.5 mg protein. Each subcutaneous dose consisted of 1 ml antigen (1 mg protein) emulsified in 1 ml Freud's complete adjuvant. The antisera samples for both *P. falciparum* and *P. cynomolgi* used in this study were the samples collected after the intravenous dose of antigens for both the rabbits.

Monoclonal antibodies

Two IgM MAbs were selected for this study, PF-1G8 produced against *P. falciparum* and PC-1E12 produced against *P. cynomolgi* schizont antigens. Ascites fluid containing these MAbs were used in a sandwich ELISA system for the detection of plasmodial antigens in human patients' sera.

Sandwich ELISA

The ELISA system used in this study was a modification of the sandwich ELISA described by Huijun *et al,* 1987. Wells of ELISA plates (Immulon 11, Dynatech) were coated with 50 µl of rabbit antiplasmodial antiserum diluted in PBS-Tween, pH 7.4 (1 in 2,500 dilution) overnight at 4°C. After washing four times with PBS-Tween, 200 µl of 1% bovine serum albumin in PBS-Tween were added and the plates incubated at room temperature (about 22°C) for 1 hour. The plates were then washed again four times with PBS-Tween and 50 µl of human patient serum (diluted 1 in 3 with PBS-Tween) were added. The plates were incubated for 2 hours at room temperature followed by washing with PBS-Tween. Fifty microliters of diluted MAb (1 in 100) were added and plates incubated for 3 hours at room temperature. After washing with PBS-Tween, 200 µl of goat serum diluted 1:100 with PBS-Tween were added and the plates incubated for another hour at room temperature. After washing with PBS-Tween, 50 µl of the enzyme conjugate, peroxidase goat anti-mouse IgM (Cappel Laboratories, USA) diluted 1 in 5,000 with PBS-Tween were added. The plates were then incubated for 1 hour, washed with PBS-Tween and 50 µl of the enzyme substrate (20 mg orthophenylene diamine in 50 ml buffer, pH 5 consisting 12.15 ml of 0.1 M citric acid, 12.85 ml of 0.1 M Na_2HPO_4 and 25 ml distilled water, and 20 µl of 30% hydrogen peroxide) were added. The plates were then incubated for 30 minutes in the dark at room temperature and the reaction stopped by adding 25 µl of 2.5 M sulphuric acid. The absorption values at 492 nm ($OD_{492\ nm}$) were read using a Dynatech ELISA reader.

The optimal dilutions of rabbit antiserum, patient's serum, MAb and conjugate were determined using a chequerboard titration. For each test, a negative, a positive and a PBS-Tween control were included. Each serum sample was tested in duplicates and the mean OD value calculated.

The test result was considered positive if the mean OD value exceeded the mean OD + 3 SD of the readings obtained with the 50 negative sera as described by Huijun *et al* (1987).

Table 1

Mean ELISA values (OD 492 nm ± SD) for various groups of human malarial sera obtained with monoclonal antibodies PF-1G8 and PC-1E12 in the sandwich ELISA for antigen detection.

Serum group	Sample size	Mean OD ± SD	
		PF-1G8	PC-1E12
Normal control	50	0.351 ± 0.036	0.205 ± 0.044
P. falciparum	30	0.733 ± 0.226	0.606 ± 0.136
P. vivax	21	0.796 ± 0.229	0.628 ± 0.219
P. malariae	13	0.755 ± 0.275	0.638 ± 0.244

Table 2

Positivity rates (%) obtained for various groups of malarial sera using monoclonal antibodies PF-1G8 and PC-1E12 in the sandwich-ELISA for antigen detection.

Serum group	Monoclonal antibody	Number Positive Number tested	% Positive
Control	PF-1G8	0/50	0
	PC-1E12	0/50	0
P. falciparum	PF-1G8	29/30	96.7
	PC-1E12	30/30	100
P. vivax	PF-1G8	21/21	100
	PC-1E12	21/21	100
P. malariae	PF-1G8	12/13	92.3
	PC-1E12	12/13	92.3

RESULTS

Table 1 summarizes the mean ELISA values (OD ± SD) obtained for the three groups of malarial sera and the control group against MAbs PF-1G8 and PC-1E12 in the sandwich ELISA. The mean OD values for the control group were 0.351 ± 0.036 for PF-1G8 and 0.205 ± 0.044 for PC-1E12. For both the MAbs, the mean OD value of the three patient groups differed significantly from the control mean for PF-1G8 ($F = 10.217$, $p < 0.05$) and PC-1E12 ($F = 31.679$, $p < 0.05$). However, both these MAbs did not detect any significant difference in mean OD values between the three groups of malaria sera ($p > 0.05$) using the Least Significant Difference test.

The positivity rates obtained using the two MAbs for the detection of circulating soluble plasmodial antigens in malarial sera are shown in Table 2. Using the cut-off point of mean OD + 3 SD of the normal control group, OD values above 0.459 and 0.337 were considered positive in the ELISA using PF-1G8 and PC-1E12, respectively. From the results obtained, PF-1G8 was able to detect soluble plasmodial antigens in 62 out of the 64 malarial sera tested, giving a sensitivity of 96%. PC-1E12 detected soluble plasmodial antigens in 63 out of the 64 sera; the sensitivity being 98%.

Both MAbs did not detect plasmodial antigens in any of the 50 normal sera tested; thus the specificity of this ELISA with both MAbs was 100%. For the *P. falciparum* group, three of the patients had only the sexual stage (gametocytes) in their blood films but were still positive for plasmodial antigens detectable by MAb PC-1E12. Monoclonal antibody PF-1G8 however, gave positive ELISA values for two of these sera.

There was poor correlation between the log number of parasites per µl blood and the mean OD values of the individual test sera for the three groups of malaria infections. With MAb PF-1G8, r = 0.05, 0.06 and 0.11 for *P. falciparum, P. vivax* and *P. malariae* infections respectively. Similarly, with MAb PC-1E12, r = 0.07, -0.05 and 0.01 for *P. falciparum, P. vivax* and *P. malariae* infections, respectively.

DISCUSSION

Plasmodial antigens that are detectable by serological methods for diagnostic and epidemiological purposes include soluble antigens in the serum of patients during or shortly after infection as well as the particulate parasite antigens within or on the erythrocyte surface. Recent work by several researchers have demonstrated that DNA probes and MAbs are more sensitive and specific tools for antigen detection. A number of *P. falciparum* probes (Barket *et al,* 1986; Franzen *et al,* 1984; Holmberg *et al,* 1986; Scaife *et al,* 1986) compared favorably in terms of specificity and sensitivity to results obtained with light microscopy. Parasitemias of 0.001% or less were detectable by these DNA probes and no cross-reactions were seen with other human plasmodia or human DNA. However, a major disadvantage of the existing DNA probes is the need to use radioactively labeled DNA. Thus alternative methods of labeling are being developed to circumvent the hazards and difficulties of handling radioactive materials.

Monoclonal antibodies employed in an ELISA system may also provide useful tools for antigen detection since ELISA is relatively simple and can be automated. In this study, MAbs PF-1G8 produced against *P. falciparum* and PC-1E12 against *P. cynomolgi* were able to detect soluble plasmodial antigens in sera of patients infected with either *P. falciparum, P. vivax* or *P. malariae.* Their ability to detect heterologous antigens is not surprising as earlier studies (unpublished data) have shown that both these monoclonal antibodies reacted with soluble antigens of *P. falciparum, P. cynomolgi* and *P. inui* in the ELISA as well as counter-immunoelectrophoresis against both the trophozoite and schizont stages, thus indicating the presence of common or similar antigenic epitopes in these plasmodial species. The extent of antigenic cross-reactivity between related species of plassmodia indicates that plasmodial antigens are serologically not species-specific. Wide cross-reactivity between simian and human malarias have been demonstrated by the indirect fluorescent antibody test (El Nahal, 1967), ELISA (Voller *et al,* 1975) and the indirect hemagglutination test (Matthews and Dilsworth, 1976).

The specificity of this assay with MAbs PF-1G8 and PC-1E12 was 100% as none of the normal sera tested was positive in the ELISA. The sensitivity rates obtained with PF-1G8 and PC-1E12 were 96%, and 98%, respectively. The sensitivity of these tests may be further improved by the purification of both the rabbit hyperimmune antisera and ascites of these monoclonal antibodies.

MAb PC-1E12 gave positive ELISA values with three sera of patients with only *P. falciparum* gametocytes in their blood films while PF-1G8 detected antigens in 2 out of the 3 sera. This finding suggests that the two MAbs may be sensitive enough to detect antigenemia in sera of patients who no longer have detectable asexual or have only sexual parasites in their peripheral blood. Also, soluble antigens associated with parasite levels as low as 32 parasites per µl blood could still be detected; thus this assay may be useful as a seroepidemiological tool. MAb PC-1E12 appeared more sensitive than PF-1G8; ELISA values for individual test sera suggest that the two MAbs may be recognizing similar but different antigenic epitopes.

No correlation was seen between the levels of parasitemia and the mean OD values of individual sera. It may be due that as the infection progressed, antibodies produced against the plasmodial antigens formed immune complexes resulting in the clearance of the antigens from the blood circulation. A similar finding was reported in mice infected with *P. yoelii* whereby the antigenemia did not correlate with the level of parasitemia as infection progressed

(Taylor *et al,* 1986).

The present study demonstrated that the two MAbs are potentially useful for plasmodial antigen detection in sera of malaria patients. Further studies should therefore be carried out to develop a more suitable ELISA system for field use example, a Dot ELISA which will obviate the need for a spectrophotometer. In addition, the present study was based on a rather small number of serum samples from known malaria patients. There is therefore a need to test these monoclonals against field samples in a blind trial.

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