Two-Site Immunoradiometric Assay for Detection of Plasmodium falciparum Antigen in Blood Using Monoclonal and Polyclonal Antibodies

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Three systems of immunoradiometric assays (IRMAs), a two-site monoclonal antibody sandwich IRMA (MAB-IRMA), two-site polyclonal antibody-monoclonal antibody sandwich IRMA (PAB-MAB-IRMA), and two-site polyclonal antibody sandwich IRMA (PAB-IRMA), were developed to detect low-grade infections with Plasmodium falciparum. The assays showed good correlation with parasitemia when tested against parasites from in vitro cultures (r = 0.996, 0.994, and 0.998 for MAB-, PAB-MAB-, and PAB-IRMA, respectively), with the ability to detect as few as 0.24, 0.67, and 1.82 parasites per 10^7 erythrocytes, respectively. The assays were specific for P. falciparum, since a serially diluted specimen from a patient with vivax malaria with an initial parasitemia of 0.8% and almost all of the undiluted specimens from five other vivax malaria patients were negative. The assays were performed on patients with falciparum malaria before and after treatment with antimalarial drugs. Before treatment, all 24 patients were positive by all three systems of two-site sandwich IRMAs. Two weeks after treatment, 81.8% (18 of 22) of the patients were positive by microscopic examination, but the IRMA positivity rates were 90.9% (20 of 22), 86.4% (19 of 22), and 81.8% (18 of 22) for MAB-, PAB-MAB-, and PAB-IRMA, respectively. Four weeks after treatment, all 19 patients were negative by microscopic examination, but 52.6% (10 of 19) of the patients were still positive with MAB- and PAB-MAB-IRMA and 31.6% (6 of 19) were positive with PAB-IRMA. Comparison between the three systems of IRMA showed that the MAB-IRMA was superior to the other two systems for three reasons. First, it gave a lower count when tested with blood from healthy individuals. Second, it gave a higher count when tested with blood from patients with falciparum malaria. Third, it gave better correlation with parasitemia when blood from falciparum malaria patients was tested. MAB-IRMA is recommended for use for the detection of low-grade P. falciparum infection.

Demonstration of antigens is becoming increasingly popular in the diagnosis of parasitic diseases because they are more closely related to present illness than are antibodies which can be detected even when the etiologic agent has disappeared. Diagnostic tests based on antigen detection have been developed for several parasitic diseases, including amebiasis (9, 15), giardiasis (8, 16), toxoplasmosis (1), and schistosomiasis (6). Detection of sporozoites of Plasmodium falciparum and Plasmodium vivax in infected mosquitoes has been done successfully by immunoradiometric assay (IRMA) or enzyme-linked immunosorbent assay (4, 5, 20, 21). The presence of blood-stage antigen in P. falciparum-infected erythrocytes has been demonstrated with a competitive binding radioimmunoassay (RIA) using polyclonal antibodies (PAB) with a sensitivity of detecting one to eight parasites per 10^7 erythrocytes (2, 11, 12).

We have recently developed a competitive binding RIA with anti-P. falciparum immunoglobulin G (IgG) prepared from people living in an area where malaria is endemic in Thailand and IgG from a monoclonal antibody (MAB) against blood stages of P. falciparum prepared in our laboratory, with sensitivities of detecting 13 and 2.2 parasites per 10^6 erythrocytes, respectively (10). We also showed that our MAB bound to the parasite antigen more specifically than did the polyclonal IgG (PlgG). The competitive-binding RIA appears to be less sensitive than microscopic examination by an experienced microscopist, who could detect as few as one parasite per 5 x 10^6 erythrocytes (14). The objectives of the present study were to develop a two-site sandwich IRMA, which is considered a more sensitive method for detection of antigen than the competitive-binding RIA, using MAB and PAB and to select the assay system for future use by comparing the results with MAB, PAB, and a combination of the two.

MATERIALS AND METHODS

Subjects. (i) Patients with falciparum malaria. Twenty-four falciparum malaria patients admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Bangkok, from June to October 1984 were studied. Blood samples were obtained from all patients during the pretreatment period, from 22 patients for a second time during the second week after treatment, and from 19 patients for a third time during the fourth week.

(ii) Patients with vivax malaria. Included in the study were a patient with vivax malaria (0.8% parasitemia) admitted to the Hospital for Tropical Diseases and five other vivax malaria patients residing at Pob Phra, Mae Sod District, Tak Province, 460 km northwest of Bangkok.

(iii) Healthy controls. Thirty healthy persons residing in Bangkok, where malaria is not endemic, were studied. They denied travelling to any endemic area in the past 2 years and hence would be most unlikely to have been exposed to malaria during the time of study.

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**Blood samples.** Three milliliters of blood was collected in screw-cap tubes containing 5 mM EDTA, from which two thick and two thin blood films were made on clean microscopic slides, stained with Giemsa, and examined by light microscopy. For the thick film, 10 μl of blood was used. The number of infected erythrocytes per 10^7 erythrocytes was determined by counting the number of parasitized cells in the thin blood smear per 10^6 erythrocytes; for the thick blood film, the entire smear was examined (100 oil immersion fields in approximately 3 min), and the number of parasites was counted and expressed per 10^7 erythrocytes, with the assumption that a Thai patient with falciparum malaria had on the average 4 × 10^6 erythrocytes per μl.

The blood collected in the tubes was centrifuged at 500 × g for 10 min at room temperature to remove the plasma, and the remaining packed cells were washed twice by centrifugation with phosphate-buffered saline, pH 7.2 (PBS; 0.01 M phosphate buffer, 0.14 M NaCl). The final cell suspension was centrifuged at 9,980 × g for 5 min at room temperature, and the packed cells were stored at −70°C until used. The packed cells were thawed, resuspended in 9 volumes of borate-buffered saline (BBS; 0.025 M disodium tetraborate, 0.1 M boric acid, 0.075 M sodium chloride, pH 8.6) containing 0.5% Nonidet P-40 (BDH Chemicals Ltd., Poole, England) (BBS-NP40) for 10 min at room temperature by the technique of Renu Dayal (World Health Organization Immunology Research and Training Centre, Faculty of Medicine, University of Geneva, Geneva, Switzerland; personal communication).

**Standard antigen.** The SO strain of *P. falciparum* was used (17). The parasites were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% heat-inactivated AB serum by the technique of Trager and Jensen (18). The culture was harvested when parasitemia reached 16.5%, of which approximately 50% were ring forms. The cells were washed twice in RPMI 1640 without serum by centrifugation at 500 × g for 10 min at room temperature. The packed infected erythrocytes were then treated with BBS-NP40 as described above. Before use, infected-erythrocyte extract was adjusted to give an initial concentration equivalent to 10^5 infected cells in 10^7 erythrocytes and serially diluted 1:5 eight times to a final dilution of 1:390,625.

**Control antigen.** Normal erythrocytes from healthy individuals were washed three times in PBS by centrifugation at 500 × g for 10 min at room temperature, and the plasma as well as theuffy coat layer was removed. The washed cells were lysed in 9 volumes of BBS-NP40 at room temperature, followed by centrifugation at 9,980 × g for 10 min at room temperature to remove nuclei and cell debris. The supernatant was then used as the control antigen and as the diluent for the standard parasite antigen (11).

**Preparation of anti-*P. falciparum* antibody.** (i) PAb. A rabbit was initially immunized subcutaneously at multiple sites with 1 ml of a suspension of 10^9 parasites of the SO strain of *P. falciparum*, of which most were in the late trophozoite and schizont stages, in physiological saline (0.85% NaCl solution) incorporated in an equal volume of Freund complete adjuvant. This was followed by three other monthly injections of 1 × 10^9, 4 × 10^9, and 4 × 10^10 parasites, respectively, in Freund incomplete adjuvant. The animal was bled by cardiac puncture 3 weeks after the last injection. The serum was inactivated by heating at 56°C for 30 min and was absorbed by mixing 1 ml of the serum with 4 ml of packed group AB erythrocytes from healthy individuals, incubated at 37°C for 30 min and at 4°C overnight, followed by centrifugation at 500 × g for 10 min at room temperature, and the supernatant was kept at −70°C until used. (ii) MAb. The F2W22C1 MAb prepared from the culture supernatant was used. This MAb was produced by fusion of Sp2/0 myeloma cells with spleen cells from a BALB/c mouse immunized with the SO strain of *P. falciparum* as described previously (10). It reacted in the indirect fluorescent-antibody test with the ring as well as all other stages of *P. falciparum* in 27 of 31 isolates tested (10).

**Radioiodination of anti-*P. falciparum* IgG.** IgG fractions from either PAb or MAb against *P. falciparum* were prepared by protein A-Sepharose 4B (Pharmacia) chromatography by the technique provided by the manufacturer. Two hundred micrograms of IgG, determined by a spectrophotometric method (19), was labeled with 2 mCi of carrier-free 125I (Amersham, England) by the iodogen tube method (13). The percent incorporation and specific activity were 97.69% and 4.2 × 10^6 cpm/μg of protein, respectively, for labeled PIgG and 95.44% and 9.6 × 10^6 cpm/μg of protein, respectively, for monoclonal IgG (MIgG). The labeled IgG was kept in small portions in PBS (pH 7.2) containing 1% bovine serum albumin (BSA) fraction V (RIA grade; US Biochemical Corp.) at −70°C and thawed once only, just before use. The labeled IgG solution was centrifuged at 9,980 × g for 10 min at room temperature to remove any formed aggregates, further diluted with BBS containing 0.5% BSA, and used at a concentration of 0.1 and 0.4 μg of protein per 30 μl for MIgG and PIgG, respectively.

**IRMA.** Wells of 96-well U-bottom assay plates (Falcon 3911 Microtect III; Becton Dickinson and Co., Paramus, N.J.) were each coated with 50 μl of MIgG or PIgG in 0.05 M sodium bicarbonate buffer, pH 9.6, at a concentration of 10 μg/ml, followed by incubation at 37°C for 3 h or at 4°C overnight. The plates were washed three times with BBS-0.5% BSA, saturated with 100 μl of BBS containing 1% BSA (BBS-1% BSA), and kept for 1 h at room temperature and at 4°C thereafter until used. The plates were thoroughly washed with BBS-0.5% BSA to which 30 μl of either the erythrocyte preparations to be tested or standard antigen in various dilutions was added to each well, followed by incubation for 3 h at room temperature. The plates were washed twice with BBS-0.5% BSA containing 0.05% Tween 80 and once with BBS-0.5% BSA. After washing, 30 μl of [125I]MIgG or [125I]PIgG (approximately 5 × 10^5 to 10^6 cpm) in PBS-1% BSA and 10% normal human serum were added, and the plates were incubated for another 3 h at room temperature. After being washed three times with BBS, the wells were cut out and placed directly into counting tubes, and bound radioactivity was measured in a gamma counter (Mini-Array type 6-20; Mini Instruments Ltd., London, England). Three systems of IRMA were developed: (i) a two-site MAb sandwich IRMA (MAb-IRMA), in which the plates were coated with unlabeled MIgG and detected with labeled MIgG; (ii) a two-site PAb-MAb sandwich IRMA (PAb-MAB-IRMA), in which the plates were coated with unlabeled PIgG and detected with labeled MIgG; and (iii) a two-site PAb sandwich IRMA (PAb-IRMA), in which the plates were coated with unlabeled PIgG and detected with labeled PlgG. All assays were done in duplicate, with an intrarun difference of less than 5%, and the means (± standard deviation [SD]) of the coefficient of variation for 12 samples in four different runs were 10.2 ± 3.1% (range, 5.2 to 14.9%); 9.1 ± 4.6% (range, 2.96 to 16.6%); and 10.12 ± 3.3% (range, 5.45 to 16.5%) for MAb-, PAb-MAb-, and PAb-IRMA, respectively.

**Statistical analysis.** Regression analysis was used to study
RESULTS

Sensitivity and specificity of IRMA. The assay performed on blood samples from 30 healthy individuals showed that the mean in cpm (MLC) ± SD was 6.2032 ± 0.194, 6.6113 ± 0.133, and 7.209 ± 0.130 cpm for MAb-, PAb-MAb-, and PAb-IRMA, respectively. A sample was considered positive if its binding was above the MLC + 4 SD of 6.9792 for the MAb-IRMA, 7.1433 for the PAb-MAb-IRMA, and 7.729 for the PAb-IRMA (equivalent to 1.074, 1.265, and 2.273 cpm, respectively).

The cutoff levels in the above three systems were used to determine the sensitivity of IRMA when various dilutions of P. falciparum-infected erythrocytes from in vitro cultures were allowed to react with antiplasmodial antibodies. The sensitivity of the IRMA was 0.24, 0.67, and 1.82 parasites per 10^7 erythrocytes with MAb-, PAb-MAb-, and PAb-IRMA, respectively (Fig. 1).

The specificity of the assay was determined in a serially diluted suspension of P. vivax-infected erythrocytes from one patient with vivax malaria with an initial parasitemia of 0.8% (Fig. 1) and in undiluted blood samples from five other vivax malaria patients, one of whom was asymptomatic (Table 1). Binding was below the cutoff level for each system in all cases except one (patient 3), in which the PAb-MAb-IRMA binding activity (7.2269 in cpm) was a little above the cutoff MLC level of 7.1433 in cpm.

Application of IRMA to clinical specimens. The blood from malaria patients collected before treatment and at various times thereafter was tested in duplicate only once with all three systems of IRMA, and the results were compared with those found by microscopic examination. All 24 patients before antimalarial treatment were positive, with MLC of 10.23 ± 0.58 (27.723 cpm; range, 6.002 to 46.879), 9.95 ± 0.54 (20.952 cpm; range, 5.640 to 35.850), and 9.66 ± 0.64 (15.678 cpm; range, 1.569 to 28.998) with MAb-, PAb-MAb-, and PAb-IRMA, respectively. In the second week after treatment, 18 of 22 patients (81.8%) were positive by microscopic examination but 20 (90.9%), 19 (86.4%), and 18 (81.8%) patients were positive by MAb-, PAb-MAb-, and PAb-IRMA, respectively. The MLC declined to 9.08 ± 1.21 (8.778 cpm; range, 725 to 26,827), 8.07 ± 1.13 (7.480 cpm; range, 994 to 25,274), and 8.67 ± 0.69 (4,915 cpm; range, 1,503 to 12,348) with MAb-, PAb-MAb-, and PAb-IRMA, respectively. In the fourth week after treatment, all 19 patients tested were negative by microscopic examination, but 10 (52.6%) were still positive by MAb- and PAb-MAb-IRMA and only 6 (31.6%) were positive by PAb-IRMA. The MLC were 7.72 ± 1.12 (2,252 cpm; range, 715 to 36,240), 7.67 ± 0.95 (2,143 cpm; range, 843 to 23,568), and 7.59 ± 0.47 (1,978 cpm; range, 1,089 to 6,272) for MAb-, PAb-MAb-, and PAb-IRMA, respectively. The relationship between parasite count and MAb-IRMA binding activity before and after treatment is presented in Fig. 2. When parasitological counts for all samples positive by microscopic examination were plotted against IRMA binding activities, a positive correlation was demonstrated (r =

![FIG. 1. Sensitivity of MAb- (-----), PAb-MAb- (---), and PAb-IRMA (- - - - -) in detecting P. falciparum-infected erythrocytes from in vitro culture and P. vivax-infected erythrocytes from one patient with vivax malaria. Based on the cutoff MLC of 6.9792, 7.1433, and 7.729 for MAb-, PAb-MAb-, and PAb-IRMA, respectively, the sensitivity was -1.4, -0.4, and 0.6 In parasites, which is equivalent to 0.24, 0.67, and 1.82 parasites per 10^7 erythrocytes for MAb-, PAb-MAb-, and PAb-IRMA, respectively.](http://jcm.asm.org/)

![FIG. 2. Parasitemia (ln parasites per 10^7 erythrocytes) and MAb-IRMA binding activity (ln cpm) in blood from patients with falciparum malaria before and 2 and 4 weeks after antimalarial treatment with healthy controls.](http://jcm.asm.org/)

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Initial parasitemia (no. of parasites/10^7 erythrocytes)</th>
<th>MLC-IRMA binding activity (ln cpm)</th>
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<tr>
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TABLE 1. Detection of P. falciparum-infected erythrocytes in patients with vivax malaria by two-site MAb-, PAb-MAb-, and PAb-IRMA
FIG. 3. Correlation between parasitemia (ln parasites per 10^7 erythrocytes) detected by microscopic examination in patients with falciparum malaria and MAb-IRMA binding activity (ln cpm) (r = 0.886).

0.886, Fig. 3). With the two other assay systems (data not shown), similar findings were observed, but with a lower degree of correlation (r = 0.803 and 0.638 for PAb-MAb- and PAb-IRMA, respectively).

DISCUSSION

Detection of low-grade parasitemia has recently been a subject of intense investigation by RIA, enzyme immunoassay, and DNA probes (2, 3, 7, 11, 12). The highest sensitivity of the assays ranged from 1 to 10 parasites per 10^6 erythrocytes. This level of sensitivity was considered by Meuwissen (14) to be lower than the level of parasitemia detected by an experienced microscopist, who in a 3-min examination (100 oil immersion fields) could identify as few as one parasite per 5 x 10^5 erythrocytes. In an attempt to improve the sensitivity of the antigen detection assay, we first developed a competitive-binding RIA with either MAb or PAb in the assay to yield, at best, the sensitivity of detecting only 2.2 parasites per 10^6 erythrocytes (10). In the present study, three systems of two-site sandwich IRMAs, MAb-, PAb-MAb-, and PAb-IRMA, were developed with the ability to detect as few as 0.24, 0.67, and 1.82 parasites per 10^7 erythrocytes, respectively. Our assays were specific, since they were positive only with P. falciparum-infected erythrocytes and negative when erythrocytes from 30 healthy individuals and P. vivax-infected cells were tested. Crossreactivity against Plasmodium malariae or Plasmodium ovale has not been determined, mainly because of the rarity of infections with these two species in Thailand. Our assays showed good correlation with parasitemia when tested against P. falciparum-infected erythrocytes from in vitro culture as well as from clinical specimens, especially when the MAb-IRMA was used (r = 0.998 and 0.886, respectively). It was observed that in most patients with parasitemia of more than 44,000 parasites per 10^n erythrocytes (0.44%), the radioactive binding did not exceed 10.7579 in cpm (47,000 cpm). This apparent lack of increased binding with a level of parasitemia beyond 0.44% was probably due to exhaustion of the radiolabeled antibody, making it unable to react with the excess number of parasites over the maximum limit of the assay.

Comparison between the three assay systems used in this study showed that the MAb-IRMA was superior to the other two assay systems for three reasons. First, it gave the lowest MLC (6.20) when tested with erythrocytes from healthy individuals, whereas those assayed with the PAb-MAb- and PAb-IRMA showed a higher MLC (6.61 and 7.20, respectively). Second, it correlated more closely with parasitemia in clinical specimens (r = 0.886) than the PAb-MAb- and PAb-IRMA (r = 0.803 and 0.638, respectively). Third, it gave higher radioactive counts with P. falciparum-positive specimens than the other two systems. It is therefore recommended that the MAb-IRMA be used in future trials with clinical specimens and in the field.

The nature of the antigen detected with our assay systems has not been determined. Neither do we know whether the antigen detected is associated with living or dead parasites or soluble parasite products. The high IRMA-positive rates for patients 4 weeks after treatment, when parasites were no longer detected by microscopic examination, suggested that the parasite molecules reacting with our MAb or PAb were present even when they were degenerated and thus could not be recognized even by experienced microscopists. Our IRMA is therefore inferior to parasitological examination in the assessment of therapeutic cure, but it is more practical than parasitological examination in a large-scale survey of specimens collected from the field, especially when parasitemia is low.

The MAb-IRMA developed in our laboratory could have wide applications, including field monitoring of the malaria control program, assessing the effectiveness of field trials of a future malaria blood-stage vaccine, detecting early recrudescence of malaria after chemotherapy, and screening blood donors. The radioisotope technique used in this study may, at present, preclude the assays from field use, but it could be substituted for the enzymatic techniques, which are we planning to develop in the near future.

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LITERATURE CITED