INFECTION AND IMMUNITY, Nov. 1987, p. 2604–2611
0019-9567/87/112604-08$02.00/0

Vol. 55, No. 11

Demonstration of Antigenic Polymorphism in *Plasmodium vivax* Malaria with a Panel of 30 Monoclonal Antibodies

PREETHI V. UDAGAMA,[1] PETER H. DAVID,[2] J. S. M. PEIRIS,[3] Y. G. ARIYARATNE,[3] K. L. R. L. PERERA,[1] AND KAMINI N. MENDIS[1]*

Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka[1]; Unite d'Immunoparasitologie, Institut Pasteur, Paris 75724, France[2]; and Department of Microbiology, Faculty of Medicine, University of Peradeniya, Sri Lanka[3]

Received 31 March 1987/Accepted 31 July 1987

A panel of 30 monoclonal antibodies was established against asexual erythrocytic stages of *Plasmodium vivax* and used to investigate the antigenic composition of the parasite. At least 38 different antigenic polypeptides of *P. vivax* were characterized by the Western blot technique. The possible location of these antigens, as well as their stage and species specificity, was determined on the basis of the staining patterns produced by these antibodies on air-dried parasites in the indirect immunofluorescence test. Immunofluorescence performed with 30 different monoclonal antibodies on 50 different isolates of *P. vivax* obtained from patients showed that a high level of antigenic polymorphism prevailed in *P. vivax*. Only six monoclonal antibodies reacted with epitopes that were represented in more than 80% of parasite isolates, and therefore, appeared to be relatively conserved among different isolates. The other 24 monoclonal antibodies reacted with only 20 to 70% of parasite isolates.

As more knowledge on malarial antigens is acquired, there is increasing evidence highlighting the importance of antigenic polymorphism in plasmodia. Antigenic variation, which has been shown to occur on the surface of infected erythrocytes in several *Plasmodium* species, *P. knowlesi* (3), *P. falciparum* (14), and *P. fragile* (9a), is only one of the mechanisms which contributes to antigenic polymorphism. Antigens can be naturally polymorphic, such as the S antigen in *P. falciparum*, (30), the major schizont surface protein Pf195 (20), the molecule thought to mediate cytoadherence (18) or certain gamete surface antigens (7). Polymorphism can also be artificially induced through splenectomy of the host (14), prolonged parasite culture (28), or immune pressure following vaccination (5). Antigenic polymorphism may hinder the development of an effective vaccine against malaria, since vaccine-induced protection will have to be effective against a wide spectrum of parasite populations.

Although *P. vivax* is one of the major pathogens responsible for malaria in humans, antigens of this parasite have only begun to be explored. The major sporozoite surface antigen (CSP) has been cloned (2, 21), and two gamete surface antigens that are involved in transmission blocking immunity have been defined through the use of monoclonal antibodies (MAbs) (21a). Preliminary characterization of a limited number of antigens of the asexual stages of the parasite has also been obtained through immune screening of a genomic *P. vivax* DNA library (5a). This relative scarcity of experimental results is partly due to the inability to maintain *P. vivax* in long-term in vitro cultures. The only source of parasites is provided by infected blood from humans or primates.

We isolated a panel of 30 MAbs directed against the asexual erythrocytic stages of *P. vivax*. This allowed us to begin investigations on the antigenic composition of this parasite and to explore the degree of polymorphism that prevailed in a series of 50 isolates obtained from infected patients.

* Corresponding author.

MATERIALS AND METHODS

Parasites. *P. vivax* asexual-stage parasites were obtained from acutely infected individuals that were admitted to the General Hospital, Colombo, Sri Lanka. The patients had synchronous infections of at least 0.15% parasitemia with predominantly early or late schizonts in the blood. Following voluntary, informed consent, up to 20 ml of blood was drawn into sterile heparinized (10 U/ml of blood) tubes and was passed through a column of prewetted CF_{11} cellulose powder (Whatman Ltd., Maidstone, United Kingdom) to remove leukocytes (13). The effluent from the cellulose column was centrifuged at 500 × *g* for 10 min and washed twice in RPMI 1640 medium (pH 7.3; (Flow Laboratories, Irvine, United Kingdom), and a 20% suspension of the washed cells was made in the same medium. Erythrocytes infected with late asexual developmental stages (trophozoites and schizonts) were separated by a single-step density gradient as described previously (16).

Extracellular female gametes of *P. vivax* were prepared from gametocyte-infected blood obtained from infected patients by previously described methods (23).

P. falciparum was also isolated from infected patients. Infected blood containing ring-stage parasites were passed through a column of CF_{11} cellulose powder to remove leukocytes, suspended at a 10% hematocrit, and cultured in vitro by the method described by Trager and Jensen (27). Parasites were harvested at the stage of mature schizonts on a single-step density gradient (24).

Three strains of *P. cynomolgi*, the Gombak, Ceylonensis, and London strains, were each inoculated into a splenectomized toque monkey (*Macaca sinica*), which is the natural host of *P. cynomolgi* in Sri Lanka. When parasitemia in infected animals reached 5 to 10%, schizont-infected blood was obtained by venipuncture and parasitized erythrocytes were separated on a density gradient as described above for *P. vivax*.

Production of MAbs. A 6-week-old female BALB/c mouse was immunized with three doses of purified parasite preparations, each of which consisted of 10^7 parasites, mainly containing schizonts with a few late trophozoites. The first immunizing dose was combined with approximately 10^8 killed organisms of *Bordetella pertussis* as an adjuvant. Immunizations were performed 4 weeks apart; the first two were administered intraperitoneally and the last was administered intravenously. Three days after the last immunizing dose, spleen cells were fused with the P3-X63-Ag8.653 (P_3U_1) myeloma cell line at a ratio of 10:1, by the method described by Galfre et al. (6) with modifications, by using 50% (wt/vol) polyethylene glycol (molecular weight, 1,450; Sigma Chemical Co., St. Louis, Mo.). At 10 to 14 days after the fusion, hybridoma clones producing antibodies to plasmodia were screened on air-dried parasites by the indirect immunofluorescence test (IFT). Selected hybrids were cloned twice by limiting dilution on thymocyte feeder layers. Spent medium from in vitro-grown cloned cultures, antibody precipitated from spent medium with 45% ammonium sulfate, and ascites fluids from pristane-primed BALB/c mice bearing the hybridomas were used as sources of MAbs. Isotypes of immunoglobulins secreted by the hybridomas were characterized by Ouchterlony double diffusion (15). This was done by using culture supernatants concentrated by ultrafiltration (stirred ultrafiltration cell; model 8010; Amicon Corp., Lexington, Mass.) reacted against affinity-purified immunoglobulin subclass-specific mouse antisera (Miles Laboratories, Inc., Elkhart, Ind.).

IFT. The IFT was performed by using air-dried parasites as antigen by the method described by Voller and O'Neill (29). Smears of purified parasites (trophozoites and schizonts or female gametes) were made on 12-well multispot microscope slides, air dried, and stored at −70°C with dessicant. Antigen smears were thawed, and each well was treated with 10 μl of antibody and incubated for 45 min at room temperature in a humid chamber. Slides were washed with phosphate-buffered saline (pH 7.2) for 15 min, stained for an additional 45 min with a 1:20 dilution of fluorescein-conjugated goat anti-mouse immunoglobulins (immunoglobulin A [IgA], IgG, and IgM, heavy and light chain specific [Cappel Laboratories, Cochranville, Pa.]), and washed for 15 min in phosphate-buffered saline. The slides were mounted in phosphate-buffered saline and examined at ×1,250 with a fluorescent microscope (Diaplan; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.). IFT was also performed by using live, unfixed extracellular gametes by a method described previously (14).

Western blotting. Purified asexual blood stages of *P. vivax* were extracted in sodium dodecyl sulfate sample buffer either with or without 5% 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, Calif.) as a reducing reagent. The proteins were electrophoretically separated on 5 to 15% sodium dodecyl sulfate-polyacrylamide gradient gels (17) and were electroeluted onto nitrocellulose paper (Bio-Rad Laboratories (26). Strips of nitrocellulose paper containing parasite proteins were blocked by incubation in Tris-buffered saline (pH 8.0) containing 5% non-fat milk powder (milk buffer), followed by incubation with spent hybridoma supernatants for 4 to 5 h at room temperature. After the strips were washed with milk buffer, they were incubated for 2 h with a 1:200 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins (Byosis, Compiegne, France), washed, and reacted with a color development reagent containing 4-chloro-1-naphthol (Bio-Rad Laboratories) to expose the sites of antigen-antibody reaction.

RESULTS

A panel of 30 MAbs which reacted with asexual erythrocytic stages of *P. vivax* by IFT and Western blots were used to characterize at least 38 different antigenic polypeptides of this parasite stage. The characteristics of these MAbs and the antigens with which they reacted are presented in Table 1.

Patterns of immunofluorescence. The MAbs were classified according to six distinct patterns observed by IFT by using air-dried asexual erthrocytic-stage parasites as antigen (Table 1). These characteristic IFT patterns are indicative of the possible location of the reactive antigen(s) either on the parasite or on or in the infected erythrocyte.

The immunofluorescent pattern designated type I appeared as fine dots distributed evenly over the entire infected erythrocyte, giving it a finely speckled appearance (Fig. 1I). These antigens could be located on or in the infected erythrocyte membrane or in the erythrocyte cytoplasm.

The type II staining pattern (Fig. 1II), which was exclusively given by a group of eight other MAbs, consisted of coarse dots superimposed on a fine speckled background over the entire infected erythrocyte; the coarse dots may represent aggregations of antigens seen in type I (Fig. 1I). This could be a derivative of type I because some MAbs produce either a type I or a type II staining pattern, depending on the isolate. Fluorescent staining of types I and II was not confined to the area over the parasite but was spread evenly on the entire infected erythrocyte.

Three MAbs grouped under type III yielded a coarse, irregularly fragmented pattern (Fig. 1III) or compact, uniform staining of the parasite, presumably depending on the stage of development of the parasite. The former pattern occurred with ameoboid trophozoites and the latter occurred with more compact parasites. These appeared to be associated with the parasite either on the parasite surface membrane or in the parasitophorous vacuole.

Seven MAbs produced a low-intensity, generalized staining (type IV) of the parasite (Fig. 1IV) that was presumably associated with the parasite surface. An intense staining around the perimeter of individual merozoites within developing schizonts was revealed by three type V MAbs (Fig. 1V). MAbs yielding type V also frequently gave the type IV staining pattern, presumably depending on the developmental stage of individual parasites; i.e., the type IV pattern represented parasites of the earlier stages of development and the type V pattern represented segmented parasites.

The type VI staining pattern (Fig. 1VI), which was produced by a single MAb, was one of distinctly demarcated pin head-like dots, numbering 4 to 18 per infected erythrocyte. This is evocative of the staining pattern that is associated in *P. falciparum* with rhoptry antigens (12, 25).

These MAbs were further distinguished by the stage specificity of the IFT reaction, which was confirmed by using parasites of different developmental stages of the intraerythrocytic cycle. Of the 30 MAbs, 11 reacted with all asexual stages (early and late trophozoites and schizonts), while the rest were directed specifically against more mature parasites (late trophozoites and schizonts; Table 1). Ring-stage parasites were not included in this investigation because of the difficulty of purifying this parasite stage on a density gradient.

Stage and species cross-reactivity. The cross-reactivity of these MAbs with sexual stages of *P. vivax* was investigated by IFT by using air-dried female gametes as the antigen. Six of these antiasexual-stage MAbs were found to also react with gametes (Table 1).

TABLE 1. Characteristics of *P. vivax* asexual erythrocytic stage MAbs

MAb	Isotype	IFT pattern[a]	Cross-reactivity[a]		Isolate specificity (%)[a,b]	M_r (10^3)
			P. vivax gametes	*P. falciparum* and *P. cynomolgi* AES[c]		
A5[d]	IgG2b	I	−	−	84	47, 92, 110, 200
A4[d]	IgG2b	I	−	−	80	112
A28[d]	IgG3	I	−	−	54	90
A3	IgG1	I	−	−	36	40[e]
A9	IgG1	I	−	−	16	54, 67, 75
A14	IgM	I	−	−	34	41
A23	IgM	I	−	−	28	40[e]
A6[d]	IgG1	II	−	−	56	74
A13[d]	IgM	II	−	−	20	43[e]
A20[d]	IgG1	II	−	−	90	54, 64, 72, 86
A10	IgG1	II	−	−	20	42
A15	IgG1	II	−	−	36	14, 29
A25	IgM	II	−	−	26	28
A32	IgM	II	−	−	20	40[e]
A34	IgG1	II	−	−	44	40
A12[d]	IgG2a	III	+[f]	−	98	23, 57
A18[d]	IgM	III	+	−	68	22, 44, 68, 74, 190
A19[d]	IgG1	III	−	−	66	26, 55, 68, 82
A16[d]	IgG1	IV	−	−	26	28
A2	IgM	IV	−	−	26	
A22	IgM	IV	−	−	24	14, 27
A24	IgM	IV	−	−	48	14, 26, 200, 225
A27	IgG2a	IV	−	−	24	
A30	IgG1	IV	+	−	22	160, 200
A31	IgM	IV	+	−	26	160, 200
A33	IgM	IV	−	−	34	28
A21[d]	IgG1	IV, V	−	+	100	30, 62, 97
A8	IgG1	IV, V	+	+[g]	94	30, 40, 57
A17	IgG2a	IV, V	+[f]	−	66	26, 74, 200
A7	IgG1	VI	−	−	62	27

[a] Air-dried parasites were used in all instances, except where indicated (*f*).
[b] Percentage of positively reactive parasite isolates of 50 tested.
[c] *P. falciparum* and *P. cynomolgi* asexual erythrocytic stages (AES).
[d] Antibodies reacted with all developmental stages (early and late trophozoites and schizonts). The others reacted with only late trophozoites and schizonts.
[e] Antigens extracted under nonreducing conditions; there was no reactivity with the reduced antigen.
[f] Antibodies also reacted with the surface of extracellular female gametes.
[g] Antibody cross-reacted only with *P. cynomolgi*.

Two of these MAbs produced a positive immunofluorescent staining of live, unfixed gametes, indicating that the antigens with which they reacted are located on the surface of extracellular gametes. Enriched gamete preparations with few contaminating asexual stages were used for the IFT. MAbs that cross-reacted with gametes positively stained nearly all gametes in the sample.

The reactivity of these anti-*P. vivax* MAbs with asexual blood stages of two other plasmodial species, *P. falciparum* and *P. cynomolgi*, was tested by IFT by using air-dried schizonts as the antigen. Of the 30 MAbs, 1 (A21) reacted with both *P. falciparum* and *P. cynomolgi* and 1 (A8) reacted with *P. cynomolgi* only (Table 1).

Antigenic polymorphism. To evaluate the extent of antigenic polymorphism of *P. vivax*, we screened by IFT 50 different parasite isolates obtained from patients using the panel of 30 MAbs. The origin of these 50 isolates was traced to 16 of the 24 different geographical districts of Sri Lanka. They were also tested with the Belem strain of *P. vivax*, which is a strain from Brazil that was raised in squirrel monkeys and that was kindly provided by J. Gysin. Positive reactivities of these MAbs with an isolate were scored (+ to ++++), depending on the intensity of the fluorescence; and in each case the proportion of parasites giving a positive reaction was noted.

The following steps were taken to ensure the validity of these results. (i) A single batch of all MAbs (culture supernatants) was used for the entire investigation. (ii) At least three different isolates were processed for the IFT and assayed simultaneously. (iii) The assay was standardized from one batch of isolates to another by including a previously tested isolate in each batch.

The results of these investigations are presented in Table 2. Six MAbs (A21, A12, A8, A20, A5, A4) were found to react with more than 80% of the parasite isolates, but the reactivity of the rest was found to be highly variable in that the MAbs reacted with only 20 to 70% of the isolates tested. It was found that when an antibody reacted with a given

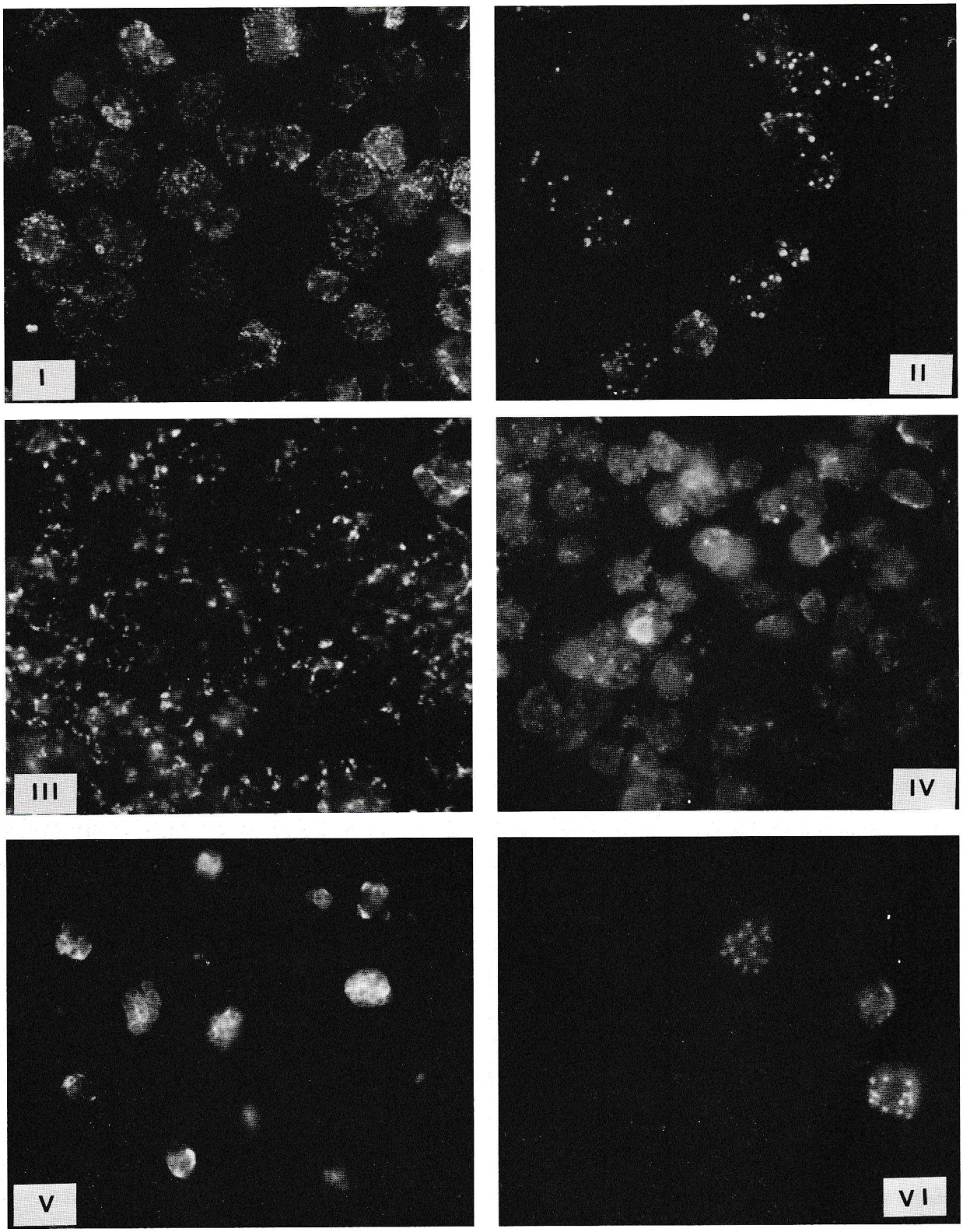

FIG. 1. Six different indirect immunofluorescent test staining patterns produced by the MAbs on air-dried asexual erythrocytic-stage parasites of *P. vivax*. Panels I to VI represent staining pattern types I to VI, respectively, as described in the text. Magnification, ×1,700.

TABLE 2. Indirect immunofluorescence reactivity of the panel of 30 anti-*P. vivax* MAbs with 50 different parasite isolates

Isolate[a]	Reactivity of the following MAbs												
	A21	A12	A8	A20	A5	A4	A18	A17	A19	A7	A6	A28	A24
A1	+	+	+	−	−	−	+	+	+	−	−	+	−
A2	+++	+++	+	+++	+++	+++	+++	−	+	−	−	+	−
A3	++	++	−	++	+	+	++	−	−	−	+	−	−
A4	++	+++	++	++	+	++	+++	+	+	+	++	+	+
A5	++	+++	++	+++	+++	+++	+++	+	−	−	+++	+	+
A6	+	+	+	++	+	+	−	−	−	−	−	+	−
A7	++	+	+	+	+++	+	+	+	+	+	−	++	+
A8	+	++	+	+	+	−	−	+	++	−	−	−	+
A9	+	+	+	+	++	−	+	++	+	−	+	−	−
A10	+	−	+	++	+	+	+	−	+	+	−	+	+
A11	++	++	+++	+++	++	++	−	+++	++	++	+	−	−
A12	+	++	++	+	−	+	+	++	+	−	+	+++	+
A13	+	+	−	+	−	−	−	−	−	+	−	−	+
Po1	++	+++	++	++	++	−	+++	+	+	+	+	++	−
Po2	+	+	+	+	++	++	+	+	−	−	−	+	−
Po3	+++	+++	+++	+++	++	+	+	+	+	+++	+	−	−
Po4	+++	++	++	++	+	+	−	++	+	+	+	−	−
Po5	+	++	+	++	+	+	+	+	+	−	+	+	−
Ku1	+++	+++	++	+	+	+	ND[b]	++	+	+	−	+	−
Ku2	++++	++++	+++	++	+	+	++++	++++	−	++	+	−	−
Ku3	+	++	++	−	+	+	−	−	++	+	+	−	−
Ma1	+++	+++	+	+++	+	+	+++	+	++	+	+	+++	+
Ma2	+	+	+	+	+	+	+	−	+	+	+	+	+
T	++	++	+	+++	+++	+++	++	++	−	−	+	−	−
Mo1	+++	++	++	−	+	+	+	++	+	+	+	+	+
Mo2	+	++	++	+	++	+	−	−	+	+	+	−	−
Mo3	+++	++	+	−	+	−	+	++	+	+	+	−	+++
Mo4	+	+	+	+++	+	+	−	+	+	+	−	++	+
Mo5	++	++	+++	+	++	++	++	++	+	+	+	++	−
Mo6	+++	++	+++	++	++	++	++	++	−	+	−	+	+
Mo7	+	++	+	++	++	++	−	−	−	−	−	++	−
C1	+	+	+	+	+	+	+	−	−	+	+	+	+
C2	+++	+	++	++	+	+	++	+++	++	+	−	+	+
C3	++	+	+	+	−	−	+	+	+	−	−	−	ND
C4	+++	++	++	++	+	+	−	−	+	+	−	−	−
B	++	++	++	++	+++	++	+++	+++	−	+	++	−	−
Ba1	+	++	−	++	+++	+	−	−	−	−	+	+	−
Ba2	++	+	+	−	−	−	+	−	−	−	−	−	−
Ka	++	+++	+	+++	+	+	++	+	−	+	+	+	−
Pu1	+++	+	+++	+++	++	++++	++	++++	+	++++	+	+	+++
Pu2	+++	+	+++	++	++	+	−	+++	+	++	+	−	+
Pu3	+	+	+	+	+	+	−	−	−	+	+	−	+
Pu4	++	+	+	+	−	−	+	+++	−	−	+	+	++
Pu5	+	+	+	+	−	+	+	+	++	−	−	−	+
R	+++	+++	++	+	+	+	++	++	+	++	−	−	+
K	+	+	++	+++	+++	++	+	+	−	ND	−	+	−
M	+++	+	++	++	+	+	++	++	++	+	+	+	−
G	+++	++	+++	+++	+	++	−	−	++	++	−	−	−
H1	+	++	+	++	++	++	−	−	+	+	−	−	+
H2	+++	++	++	++	−	−	+	−	+	−	−	+	+
FG[c]	+	++	+	+++	+	+	+	+	−	−	−	−	+

parasite isolate, it generally reacted with 80% or more of the parasites in that particular isolate.

In addition to culture supernatants, several MAbs were tested as ascites fluid at various dilutions with a series of parasite isolates. When a culture supernatant of an antibody reacted with an isolate, the ascites fluid of that MAb also reacted with that isolate up to a dilution of 1:100,000. When a MAb as culture supernatant did not react with an isolate, it failed to react even as ascites fluid at a dilution of 1:2.

DISCUSSION

MAbs were classified into six groups according to the type of staining obtained by immunofluorescence. A comparison of our results with the staining patterns obtained previously with MAbs directed against defined antigens of *P. falciparum* led us to formulate several assumptions on the location of these antigens in the parasitized erythrocyte. The fluorescence image produced by MAbs group VI is evocative

TABLE 2—*Continued*

A34	A3	A15	A33	A14	A23	A16	A31	A2	A25	A22	A27	A30	A13	A32	A10	A9
ND	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ND	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	++
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	−	−	+	+	−	−	−	+	+	−	−	−	−	−	+	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	−	−	+	+	++	+	+	+	−	+	+	+	+	−
−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−
−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−
+	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	+
−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	ND
+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ND	−	−	+	−	−	+	−	−	−	+	+	++	+	−	+	−
+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−
+	+	+	−	−	ND	−	+	−	−	+	+	−	+	−	−	−
+	+	+	+	+	+	+	−	−	+	−	−	+	−	−	+	−
+	−	+	−	+	−	+	−	−	−	−	+	−	−	+	−	−
ND	++	+	−	+	+	++	++	+	+	+	+++	++	−	+	+	−
+	−	−	+	+	−	−	+	−	+	+	+	−	+	+	−	−
ND	−	−	+	−	−	++	−	−	−	−	−	−	−	−	−	−
+	+	+	+	+	+	−	++	+	+	+	+	+	−	+	−	ND
−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	+	+
−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	++	+	+	+++	−	−	+	−	+	−	−	−	−	−	+	−
−	+	+	−	++	−	−	−	−	+	−	−	+	−	−	−	−
+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
+	+	+	+	+	−	+	−	+	−	−	+	+	+	+	−	+
−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	−	+	+	−	+	−	−	+	+	+	−	+	−	−
ND	+	++	+	+	+	+	+	+	+++	−	−	++	+	++	+	+
+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
−	−	−	−	+	−	−	+	−	−	−	+	−	−	−	−	+
−	−	−	−	−	−	+	−	+	−	−	+	−	+	−	−	−
−	−	−	−	−	+	−	−	−	−	+	−	−	+	−	+	−
+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	−	−	+	−	+	+	+	−	−	−	−	+	+	+	−	−
−	+	+	−	++	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
−	+	+	−	+	−	+	−	−	+	−	−	+	−	−	−	−

[a] Letter before an isolate number indicate a single geographical district of Sri Lanka. Isolates were obtained from 16 such districts.
[b] ND, Not determined.
[c] Belem strain of *P. vivax* from Brazil raised in squirrel monkeys.

of the staining of rhoptries. The grapelike type of fluorescence obtained with one of the MAbs, along with the molecular weight range of the corresponding antigens, suggests that these may be the equivalent in *P. vivax* of the major schizont and merozoite antigen Pf195 and of its degradation or processing products (11). More specific to *P. vivax* is the striking staining pattern of the erythrocyte cytoplasm observed with several MAbs (groups I and II); it may indeed be related to Schuffner's dots and reflect the high level of metabolic activity which appears to take place in the cytoplasm of the *P. vivax*-infected erythrocyte, as is evident from results of ultrastructural studies (1).

The sharing of some antigens between the asexual erythrocytic and sexual stages is suggested by the cross-reactivity of six MAbs with gametes. Two MAbs, (A17 and A12, one of which reacted with a major parasite polypeptide of 200 kilodaltons on the schizont and merozoite surface and the other which recognized two asexual-stage antigens of 23 and 57 kilodaltons and which appeared to also be located on the parasite surface) cross-reacted by IFT with live, unfixed

extracellular gametes. They also reacted with antigens of gamete origin with corresponding molecular weights on Western blots (data not shown), indicating that these antigens may be located on the gamete surface as well. The sharing of some antigens between these stages of the life cycle of the malaria parasite may be expected, considering that gametocytes and asexual erythrocytic stages occur almost concurrently in human hosts and share a common intraerythrocytic location. Even though anti-plasmodial immunity is considered to be broadly stage specific (4, 8, 10, 22), the finding on the parasite surface of major antigens which are shared between two different life cycle stages might justify further exploration of the possible functional significance of these antigens.

Immunofluorescence performed with 30 different MAbs on 50 different isolates of *P. vivax* obtained from patients revealed the existence of a high level of antigenic polymorphism. Only six MAbs reacted with epitopes that were represented in more than 80% of parasite isolates. Most isolates were tested several times, in each experiment leading to an identical pattern of reactivity with the battery of MAbs. Several observations indicate that our results were indeed related to antigenic polymorphism and not to differences in the developmental stages of parasites in different preparations. (i) Only schizont-containing parasite preparations were retained in this study. (ii) Differences in reactivity patterns were observed with many non-stage-specific MAbs, and thus, they could not be merely linked to the absence of a given stage in certain parasite isolates. (iii) The observation that with all isolates there were always some of the schizont-specific MAbs that gave a positive reaction confirmed that the negative reaction of other schizont-specific MAbs could not be explained by insufficient parasite development.

It is possible that a number of MAbs directed against the most highly polymorphic antigens may have been discarded at the screening stage, because screening was performed with isolates that were different from those used to immunize the mice. This problem is inherent to the use of parasites obtained from humans, as each patient represents a different parasite isolate or strain, as relatively few parasites can be obtained from a single patient, and as these parasites can neither be cryopreserved nor cultured. The degree of antigenic polymorphism evident from this study is possibly, therefore, an underestimation. The use of immunofluorescence as an indicator of reactivity could have also led to an underestimation of the degree of polymorphism. Indeed, preliminary data obtained by immunoblotting demonstrate the existence of certain antigens for a different type of polymorphism, size polymorphism, which cannot be assessed by immunofluorescence.

A given pattern of reactivity could not be linked to a given geographical origin of the parasite. The *P. vivax* parasites used in this study were obtained from patients admitted to the General Hospital in Colombo. Malaria transmission does not occur in the Colombo area. Patients contracted the disease in various other regions of the country. Although the possibility of a relapse could not be excluded, in most cases the origin of infection was related to a recent stay in a region where transmission occurred. This allowed us to map the distribution of the different isolates. Comparable levels of heterogeneity were observed whether the compared isolates originated from the same or distant regions. The panel of MAbs used in this study was also used to examine a Brazilian strain of *P. vivax* that was adapted to the squirrel monkey (*Saimiri sciureus*). MAbs that reacted with the most conserved epitopes of the Sri Lankan parasites also reacted with the Brazilian parasites; and although many MAbs did not react with the Brazilian strain, some that did not react with certain Sri Lankan isolates did react with the Brazilian strain. This indicates that patterns of antigenic polymorphism do not reflect the geographical origin of the parasite isolates (19), as is the case with *P. falciparum*.

One MAb cross-reacted with asexual erythrocytic stages of two other plasmodial species, *P. falciparum* and *P. cynomolgi*, and another cross-reacted with *P. cynomolgi* only. The epitopes with which these MAbs reacted appeared to be highly conserved even among species, and it is noteworthy that these were among the epitopes that were found to be highly conserved among different *P. vivax* isolates.

So far, several indications may lead to the impression that *P. vivax* exhibits a lower level of plasticity of the genome than does *P. falciparum*. For example, in regions where *P. falciparum* and *P. vivax* coexist, under identical drug pressure extensive drug resistance has occurred with *P. falciparum*, whereas *P. vivax* has remained fully sensitive. However, the degree of antigenic polymorphism which we have demonstrated in *P. vivax* is at least as high if not higher than that seen in *P. falciparum* (9, 19). Antigenic polymorphism may reflect, in part, the ability of *Plasmodium* species to adapt to an altered environment. A better knowledge of the mechanisms underlying such an adaptation may provide insights into the development of a vaccine, as well as to other methods of control of the disease.

ACKNOWLEDGMENTS

We are grateful to those who provided us with material: Farida Nato, Hybridolab, Institut Pasteur, Paris, for the P_3U_1 myeloma cell line; R. Gwadz and L. H. Miller, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Md., for *P. cynomolgi* strains; and Jurg Gysin, Institut Pasteur, Cayenne, French Guiana, for *P. vivax* parasites of the Brazilian strain. We are also very thankful to Sunil Premawansa of our research unit for guidance and valuable discussion and to K. G. Kapilananda and C. P. Gamage for expert technical assistance. The cooperation of M. M. Ismail and M. Pinto is gratefully acknowledged.

This investigation was supported by the United Nations Development Program/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases and by the Institut National de la Santé et de la Recherche Médicale.

LITERATURE CITED

1. **Aikawa, M., L. H. Miller, and J. Rabbege.** 1975. Caveola-vesicle complexes in the plasmalemma of erythrocytes infected by *Plasmodium vivax* and *P. cynomolgi*. Am. J. Pathol. **79**:285–294.
2. **Arnot, D. E., J. W. Barnwell, J. P. Tam, V. Nussenzweig, R. Nussenzweig, and V. Enea.** 1985. Circumsporozoite protein of *Plasmodium vivax*: gene cloning and characterization of the immunodominant epitope. Science **230**:815–818.
3. **Barnwell, J. W., R. J. Howard, H. G. Coon, and L. H. Miller.** 1983. Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. Infect. Immun. **40**:985–994.
4. **Carter, R., R. W. Gwadz, and I. Green.** 1979. *Plasmodium gallinaceum* transmission blocking immunity in chickens. II. The effect of antigamete antibodies *in vitro* and *in vivo* and their elaboration during infection. Exp. Parasitol. **47**:194–203.
5. **David, P. H., D. E. Hudson, T. J. Hadley, F. W. Klotz, and L. H. Miller.** 1985. Immunization of monkeys with a 140 kilodalton merozoite surface protein of *Plasmodium knowlesi*: appearance of alternate forms of this protein. J. Immunol. **134**:4146–4152.

5a. **David, P. H., D. F. Wirth, D. M. Mattei, L. S. Ozaki, F. G. Pons, and K. N. Mendis.** 1987. Cloning of *Plasmodium vivax* DNA using an expression vector. UCLA Symp. Mol. Cell. Biol. New Ser. **42**:691–699.

6. **Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard.** 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature (London) **266:**550–551.
7. **Graves, P. M., R. Carter, T. R. Burkot, J. Rener, D. C. Kaushal, and J. L. Williams.** 1985. Effects of transmission-blocking monoclonal antibodies on different isolates of *Plasmodium falciparum*. Infect. Immun. **48:**611–616.
8. **Gwadz, R. W., A. H. Cochrane, V. Nussenzweig, and R. S. Nussenzweig.** 1979. Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of *Plasmodium knowlesi* and characterization of surface antigens of these parasites. Bull. W.H.O. **57:**165–173.
9. **Hall, R., J. McBride, G. Morgan, A. Tait, J. W. Zolg, D. Walliker, and J. Scaife.** 1983. Antigens of the erythrocytic stages of the human malaria parasite *Plasmodium falciparum* detected by monoclonal antibodies. Mol. Biochem. Parasitol. **7:**247–265.

9a. **Handunetti, S. M., K. N. Mendis, and P. H. David.** 1987. Antigenic variation of cloned *Plasmodium fragile* in its natural host (*Macaca sinica*): sequential appearance of variant antigenic types. J. Exp. Med. **165:**1269–1283.

10. **Holder, A. A., and R. R. Freeman.** 1981. Immunization against blood-stage rodent malaria using purified parasite antigens. Nature (London) **294:**361–364.
11. **Holder, A. A., and R. R. Freeman.** 1982. Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody. J. Exp. Med. **156:**1528–1538.
12. **Holder, A. A., R. R. Freeman, S. Uni, and M. Aikawa.** 1985. Isolation of a *Plasmodium falciparum* rhoptry protein. Mol. Biochem. Parasitol. **14:**293–303.
13. **Homewood, C. A., and K. D. Neame.** 1976. A comparison of methods used for removal of white cells from malaria infected blood. Ann. Trop. Med. Parasitol. **70:**249–251.
14. **Hommel, M., P. H. David, and L. D. Oligino.** 1983. Surface alterations of erythrocytes in *Plasmodium falciparum* malaria: antigenic variation, antigenic diversity and the role of the spleen. J. Exp. Med. **157:**1137–1148.
15. **Hudson, L., and F. C. Hay.** 1980. Practical immunology, p. 117–121. Blackwell Scientific Publications, Ltd., London.
16. **Ihalamulla, R. L., and K. N. Mendis.** 1987. *Plasmodium vivax:* isolation of mature asexual stages and gametocytes from infected human blood by colloidal silica (Percoll) gradient centrifugation. Trans. R. Soc. Trop. Med. Hyg. **81:**25–28.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Leech, J. H., J. W. Barnwell, L. H. Miller, and R. J. Howard.** 1984. Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum* infected erythrocytes. J. Exp. Med. **159:**1567–1575.
19. **McBride, J. S., D. Walliker, and G. Morgan.** 1982. Antigenic diversity in the human malaria parasite *Plasmodium falciparum*. Science **217:**254–257.
20. **McBride, J. S., P. D. Welsby, and D. Walliker.** 1984. Serotyping *Plasmodium falciparum* from acute human infections using monoclonal antibodies. Trans. R. Soc. Trop. Med. Hyg. **78:** 32–34.
21. **McCutchan, T. F., A. A. Lal, V. F. De La Cruz, L. H. Miller, W. L. Maloy, Y. Charoenvit, R. L. Beaudoin, P. Guerry, R. Wistar, Jr., S. L. Hoffman, W. T. Hockmeyer, W. E. Collins, and D. Wirth.** 1985. Sequence of the immunodominant epitope for the surface protein on sporozoites of *Plasmodium vivax*. Science **230:**1381–1383.

21a. **Mendis, K. N., J. S. M. Peiris, S. Premawansa, P. V. Udagama, Y. Munesinghe, M. Ranawaka, R. Carter, and P. H. David.** 1987. Immune modulation of parasite transmission in *Plasmodium vivax* malaria. Anti-gamete antibodies can both block and enhance transmission. UCLA Symp. Mol. Cell. Biol. New Ser. **42:**417–426.

22. **Mitchell, G. H., W. H. G. Richards, G. A. Butcher, and S. Cohen.** 1977. Merozoite vaccination of douroucouli monkeys against falciparum malaria. Lancet **i:**1335–1338.
23. **Munesinghe, Y. D., K. N. Mendis, and R. Carter.** 1986. Anti-gamete antibodies block transmission of human vivax malaria to mosquitoes. Parasite Immunol. **8:**231–238.
24. **Saul A., P. Myler, T. Elliot, and C. Kidson.** 1982. Purification of mature schizonts of *Plasmodium falciparum* on colloidal silica gradients. Bull. W.H.O. **60:**755–759.
25. **Schofield, L., G. R. Buschell, J. A. Cooper, A. J. Saul, J. A. Upcroft, and C. Kidson.** 1986. A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. Mol. Biochem. Parasitol. **18:**183–195.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1976. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
27. **Trager, W., and J. B. Jensen.** 1976.Human malaria parasites in continuous culture. Science **193:**673–675.
28. **Udeinya, I. J., P. M. Graves, R. Carter, M. Aikawa, and L. H. Miller.** 1983. *Plasmodium falciparum:* effect of time in continuous culture on binding to human endothelial cells and amelanotic melanoma cells. Exp. Parasitol. **56:**207–214.
29. **Voller, A., and P. O. O'Neill.** 1971. Immunofluorescence method suitable for large-scale application to malaria. Bull. W.H.O. **45:**524–529.
30. **Wilson, R. J. M.** 1980. Serotyping *Plasmodium falciparum* malaria with S-antigens. Nature (London) **284:**451–452.