Jpn. J. Infect. Dis., 55, 150-156, 2002

Original Article

Isolation, Part Characterization, Immunogenicity, and Specificity Study of *Plasmodium falciparum* Culture Supernatant

Maung M. Mya, Arati Roy[1], Rajendra K. Saxena* and Kunal B. Roy[2]

Centre for Biomedical Engineering, Indian Institute of Technology, Hauz Khas, New Delhi,
[1]Malaria Research Centre, 22 Shamnath Marg, Delhi and
[2]Centre for Biotechnology, Jawaharlal Nehru University, New Delhi, India

(Received February 4, 2002. Accepted September 18, 2002)

SUMMARY: A *Plasmodium falciparum* malaria blood stage antigen was isolated from in vitro parasite culture supernatant. The chemical composition of the antigen was studied by high-performance thin-layer chromatography, thin-layer chromatography, gas-liquid chromatography, and other chemical methods. Such analysis indicated it to be a glycophospholipid (GPL) and to be composed of xylose, mannose, galactose, and glucose linked to a phospholipid, but no inositol. The extracted and purified antigen's sensitivity and specificity properties were assessed by laser immuno assay and enzyme-linked immunosorbent assay. The results of the sensitivity study showed a very high malaria antibody-binding response compared to other known antigens. The specificity study of GPL antigen with different nonmalarial samples showed no positive response within the limit of significance. This isolated GPL antigen appears to be better than other antigens.

INTRODUCTION

Plasmodium falciparum malaria remains as one of the most serious diseases in the world and kills millions of people each year. The disease can be controlled if it is diagnosed at early stages of development. Circulating antibodies for diagnosis of *P. falciparum* have often been used for the assessment of disease status. Recent studies have shown that the sensitivity and specificity of an antigen play a significant role in disease diagnosis (1,2). Several *P. falciparum* antigens have been identified and characterized in the recent past and are being used for detecting *P. falciparum* antibodies in human blood to help in the early diagnosis of malaria. However, all these diagnostic antigens have their own limitations in terms of specificity and sensitivity (3-9). We report here the isolation of a new antigen from *P. falciparum* culture supernatant. The specificity and sensitivity of the antigen in the diagnosis of *P. falciparum* malaria have been determined and compared with seven antigens, namely: RESA antigen, *P. falciparum* antigen, and other antigens such as LSAR, HRP-2, CSP-60, $EENV_4$, and SC-5. The specificity of the extracted antigen was examined with 10 nonmalarial sera to determine the *P. falciparum* specificity.

MATERIALS AND METHODS

Isolation and purification of glycophospholipid (GPL) antigen: A single *P. falciparum*, PSJ-M strain from Shahjahanpur, Uttar Pradesh, India was adapted for continuous culture study, as described by Trager and Jensen (10). One liter of fresh parasitized culture supernatant was centrifuged at 2000 rpm for 15 min at 4°C; the supernatant was lyophilized and then dialyzed repeatedly against water and finally extracted with a 10 chloroform: 20 methanol: 8 water mixture (11). The chloroform layer when evaporated yielded 60.4 mg of sticky product, which we refer to as GPL1. Similarly, nonparasitized culture supernatant was processed by an identical protocol and yielded GPL2.

Analysis of the chemical components: Analysis of carbohydrates, proteins, and phosphate were carried out according to a published procedure (5% phenol-H_2SO_4 test [12] for total sugar, the method described by Lowry et al. [13]: proteins, and the method described by Bartlett [14]: phosphate). The estimated results for sugar were 0.25%, and for proteins 0.035%, and with the rest of the material being phosphate and lipids. No inositol or aminosugar was found in GPL1.

Gas-liquid chromatography (GLC): GPL1 was converted as an acetylated derivative, analyzed using a GLC apparatus, and found to contain glucose, galactose, mannose, and xylose by comparison with known standard sugars. Unfortunately, GPL2 was in limited quantity to analyze. GPL1 and GPL2 components showed different mobilities on high-performance thin-layer chromatography (HPTLC) and thin-layer chromatography (TLC). The mobility and specific chemical test (iodine vapour test) (15) confirmed the presence of GPL in GPL1 and GPL2.

Comparison of GPL1 and GPL2 by enzyme-linked immunosorbent assay (ELISA): The antigen's reaction properties were tested by ELISA using GPL1 and GPL2 under identical conditions with different serum dilutions. Five each of *P. vivax*-positive, *P. falciparum*-positive, and negative control serum were used at 1:100, 1:200, 1:400, 1:800, 1:1000, and 1:10000 dilutions.

Study population: Fifty each of *P. falciparum*-positive and -negative control human blood samples were tested. Nonmalarial serums of 10 each (polio, HIV, tuberculosis, pregnancy, asthma, hepatitis B, leprosy, filariasis, leishmaniasis, and healthy persons) from All India Institute of Medical Sciences Hospital, New Delhi, India were used for the specificity study. The sensitivity and specificity of GPL1 along with other parasite antigens (RESA, *P. falciparum*, LSAR, HRP-2, CSP-60, $EENV_4$) and a nonparasite (SC-5) were used for testing immunoreactivity. RESA (a nona-peptide derived from RESA/*P. falciparum* 155 origin). Synthetic *P. falciparum* antigen (a

*Corresponding author: Tel: +91-68-64851, E-mail: rks@cbme.iitd.ernet.in

parasitized enriched, sonicated, and crude preparation of *P. falciparum* parasite). LSAR: (a synthetic liver stage parasite antigen), HRP-2: (a histidine rich protein antigen), CSP-60: (a circumsporozoite laboratory synthesized peptide), $EENV_4$: (an erythrocyte surface membrane parasite antigen originating from RESA/*P. falciparum* 155), SC-5: (a nonparasitized synthetic antigen) were all kind gifts of International Centre for Genetic Engineering and Biotechnology, Delhi, India.

Estimation of immunoreactivity by ELISA and laser immuno assay (LIA) techniques: The ELISA method has been used for determining the sensitivity and specificity of antigens and antibody reactions. ELISA was performed according to a published procedure (16). Costar ELISA plates (Costar Corporation, Cambridge, Mass., USA) were used for the immunoreaction and an ELISA reader was used for reading OD values (Diagnostic Pasteur LP300, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) at 492 nm. Conjugates and other chemicals were obtained from India.

The LIA method was performed as described earlier (17); in brief 1% polystyrene beads were coated with different types of antigens and allowed to react with specific antibodies in capillaries for micro agglutination. The intensity of scattered light was estimated by He-Ne LASER system (Aerotech, Pittsburgh, Pa., USA) operated at 632.8 nm, and the photocurrent was measured by Brookhaven instrument (model: 9000 AT digital correlater, Brookhaven, Holtsville, N. Y., USA).

RESULTS

Analysis of *P. falciparum* culture supernatant: The chemical composition of parasite culture (in vitro) supernatant end product was analyzed by TLC, HPTLC, and GLC. GPL1, which is a parasitized component of the *P. falciparum* culture supernatant, was thoroughly investigated and is shown in Table 1. GPL2, which is a parasite-free culture supernatant product, was also examined chemically. The acid hydrolysis test (phenol-sulphuric test) showed that GPL1 consists of sugar moieties, and its high solubility in chloroform and ether indicate that it is a lipid. Further, its TLC mobility indicates its primarily lipid nature. Preliminary investigation of GPL1 indicate that it is a glycolipid, and the TLC results (Table 1) confirm the presence of phosphate, but no aminosugar.

The GLC experiment using a 3% ECNSS glass capillary column and a Hewlett Packard Gas Chromatogram showed an acetylated derivative of an unknown glycolipid (GPL1), while known standard sugar derivatives, in comparison, indicated the presence of four different kinds of sugar moieties, as shown in Fig.1. Then most interesting finding is the lack of any inositol sugar, which is very common in nature. From the surface-area measurement, the sugar ratio was derived, and it was found that xylose and mannose levels were two-fold higher than those of glucose and galactose. Generally, glycophosphatidylinositol components are composed only of mannose, which is known to originate from a parasite. GPL1 is totally free of a protein component, as estimated by the method described by Lowry et al. (13).

Comparison of GPL1 and GPL2 antigens by ELISA: GPL1 and GPL2 were compared with different malariogenic and nonmalarial sera, and a clear-cut difference was observed in reaction properties. GPL1 showed specificity as well as sensitivity with the ELISA test, as shown in Fig. 2. Dilutions of the GPL1 and GPL2 antigens were made at 1:100, 1:200, 1:400, 1:800, 1:1000, and 1:10,000, and binding properties were studied by ELISA. Five *P. falciparum*-positive, five *P. vivax*-positive and five healthy control subjects pooled sera were used for detecting specificity related to GPL antigens (GPL1 and GPL2). The antigen concentration used in all assays for optimum binding was 1 μgm/ml. The comparison of GPL1 and GPL2 antigens with pooled, *P. vivax*, *P. falciparum*, and control serum showed a good correlation between the GPL1 antigen and *P. falciparum*-positive serum. GPL2 was isolated from a culture of noninfected erythrocytes, i.e., parasite-free culture supernatant was treated the same way as the GPL1. In GPL2, there was no difference observed among the three different sera with different dilutions, indicating that GPL2 does not originate from a parasite and is nonspecific for malaria antibody. The checkerboard titration for comparing antigen antibody binding showed that at 1:100 antigen dilutions, GPL1 had one OD with *P. falciparum*-positive serum while less than 0.4 OD for *P. vivax* and control serum.

Table 1. Chemical analysis of *Plasmodium falciparum* culture supernatant by different methods

Method	Content	Status
Thin-Layer Chromatography		
Iodine vapor	Glycolipid	Present
Bial's reagent (Sigma: O-7875)	Sugars	Present
Zinzadze reagent test (Sigma No. M-3389)	Phospholipid	Present
Barlett method	Phosphate	Present
2% Ninhydrin method	Aminosugar	Absent
High-Performance Thin-Layer Chromatography	Glycophospholipid	Present
Chemical Tests		
Lowry method	Protein	0.035%
5% Phenol-H_2SO_4 test	Total Sugars (Carbohydrate)	0.25%
		Mean
Gas-Liquid Chromatography	Xylose	0.35 cm^2
	Mannose	0.35 cm^2
	Galactose	0.25 cm^2
	Glucose	0.25 cm^2

[Estimation of sugar in GPL1: A sensitive area planimeter (cm^2) was used for the measurement of the area of sugar moieties in the graph: Ratio: (Xylose3: Mannose3: Galactose1.5: Glucose1.5) Total sugar = 0.25%]

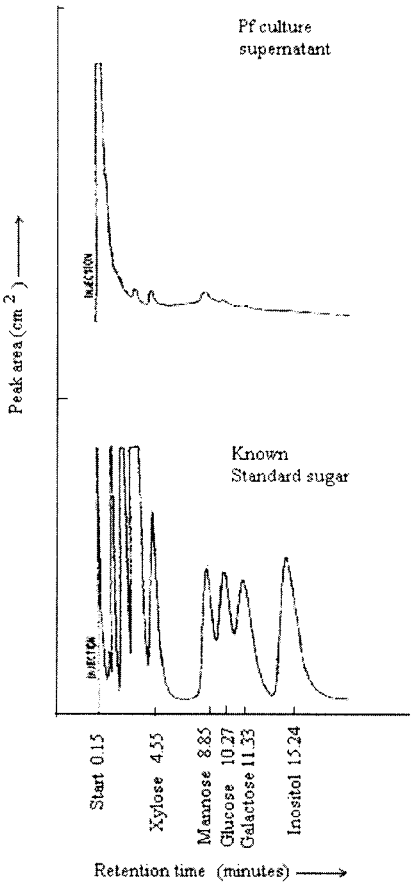

Fig. 1. Study of GPL1 malaria culture supernatant by a Hewlett Packard Glass capillary gas chromatogram (ECNSS 3%).

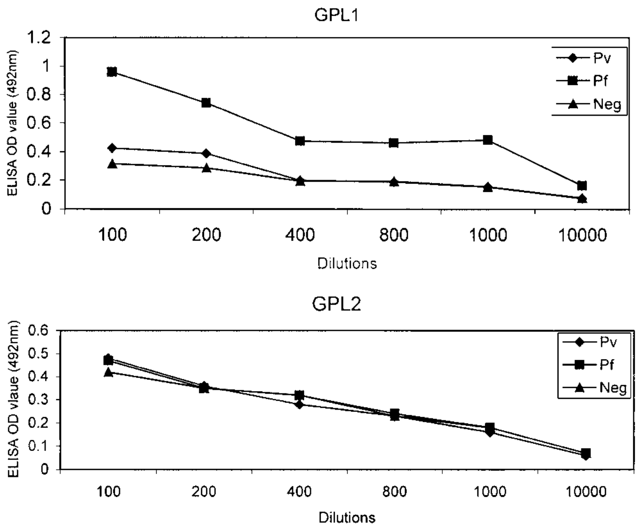

Fig. 2. Sensitivity and specificity of GPL1 and GPL2 with *P. falciparum*, *P. vivax*, and control serum by ELISA.

GPL2 had almost the same ELISA OD value (>0.5) in three groups of serum at 1:100 dilutions. These results suggest that the GPL1 antigen is of parasite origin and is hence specific to *P. falciparum*-positive infection.

Study of immunoantigenicity: (i) Sensitivity: Sera from 50 *P. falciparum*-positive cases and 50 healthy malaria-negative subjects were studied using seven malarial antigens and one nonmalarial antigen. The results shown in Table 2 emphasize the sensitivity of GPL1 compared to other antigens based on LIA and ELISA methods. Positive and negative sera with GPL1 antigen showed an almost eightfold difference in LIA, and in ELISA fourfold higher OD values were observed. In other antigens of parasite origin, synthetic peptides showed lower immunoreactivity than GPL1. In the case of nonmalarial SC-5, LIA, and ELISA values were almost identical in both positive and negative subjects. An excellent sensitivity was found with GPL1 antigen in comparison with other antigens. The LIA sensitivity results showed that GPL1 can detect 96% of positive cases of malaria, while the sensitivity of other malarial antigens ranged between 70-88% and a very low immunoactivity (10%) was seen with nonmalarial antigen (Table 2). GPL1 has also been found by ELISA to be more sensitive (88%) for detection of malaria than other antigens.

(ii) Specificity: In Fig. 3 the specificity of different antigens

Table 2. Sensitivity study of GPL1 with other antigens by LIA and ELISA methods

S. No.	Particulars (*n*=50)	GPL1		RESA		Pf		LSAR		HRP-2		CSP-60		EENV$_4$		SC-5	
		LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)	LIA (nm)	ELISA (OD)
1	*P. falciparum*																
	Mean	804.96	0.79	551.51	0.56	492.54	0.54	461.88	0.50	457.51	0.47	450.71	0.45	397.87	0.34	126.00	0.28
	±SD	182.23	0.13	272.64	0.21	223.29	0.21	218.57	0.12	206.25	0.17	198.76	0.07	187.17	0.05	21.68	0.05
	+ve subjects	48	44	39	33	44	35	39	34	43	37	37	35	35	33	5	5
	(% +ve)	96%	88%	78%	66%	88%	70%	78%	68%	86%	74%	74%	70%	70%	66%	10%	10%
2	Control																
	Mean	98.36	0.20	98.72	0.15	97.13	0.16	97.71	0.19	97.71	0.10	97.24	0.17	93.45	0.10	96.77	0.16
	±SD	3.85	0.02	4.89	0.06	3.45	0.05	4.28	0.05	4.28	0.04	3.47	0.05	4.38	0.06	4.46	0.05
	False +ve	0	0	1	4	1	3	2	3	0	1	2	4	1	3	0	1
		0%	0%	2%	8%	2%	6%	4%	6%	0%	2%	4%	8%	2%	6%	0%	2%
	Cut of value (mean ± 2SD)	106.07	0.24	108.5	0.26	93.98	0.25	107.77	0.29	105.69	0.26	104.18	0.26	106.22	0.17	105.69	0.26
	P value			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	t-test			5.47	4.93	7.87	5.38	8.53	6.89	8.93	7.38	8.37	8.15	9.58	10.87	23.99	13.98

GPL1 antigen=Glycophospholipid antigen; RESA/Pf155=ring infected erythrocyte surface antigen (AR1); Pf antigen=*P. falciparum* enriched sonicated soluble antigen; LSAR=Liver stage protein; HRP-2=Histidine rich protein-2; CSP-60=Circumsporozoite surface protein; EENV$_4$=Tetramer from RESA/Pf155; SC-5=Non malarial peptide.

against malarial and nonmalarial patient's serum by ELISA is shown. At least seven different antigens other than GPL1 were found to have no immunoreactivity with other nonmalarial sera. The only observable difference was found with *P. falciparum* serum and seven different parasite antigens. GPL1 showed the highest ELISA OD value (1 OD) compared with the nonmalarial antigen SC-5 (OD value 0.2). The specificity of GPL1 was found to be the best in comparison with the rest of the antigens. A much improved specificity was observed when the same serum and the same antigens were used with the LIA method, as shown in Fig. 4. One important finding with the LIA method was an eightfold difference in the

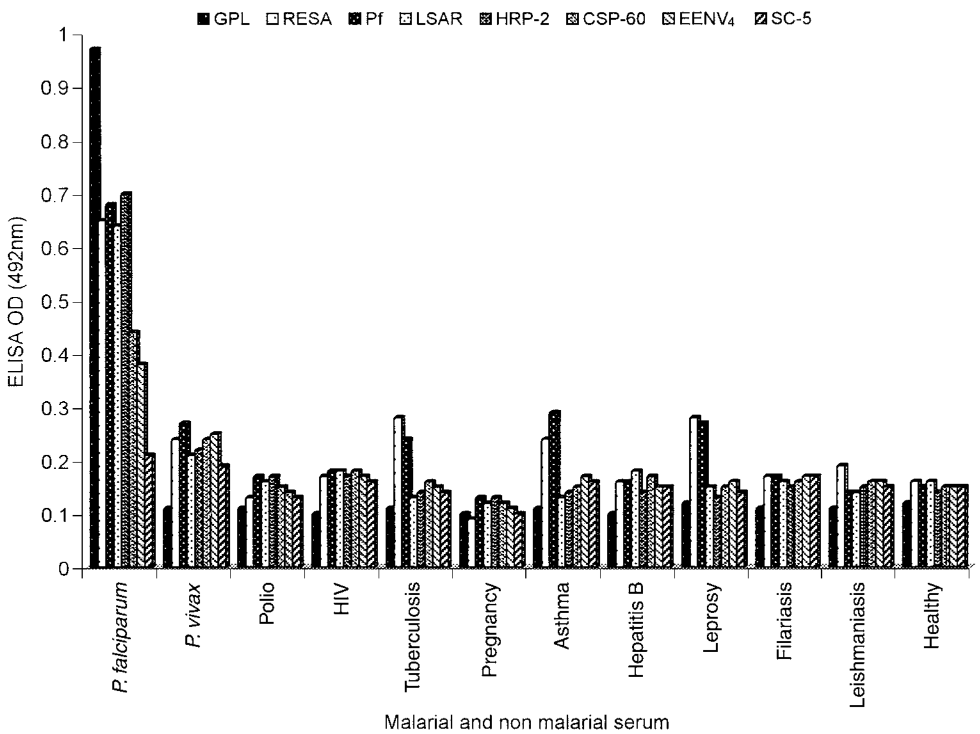

Fig. 3. Specificity of different antigens against serum of malarial and nonmalarial patients by ELISA.

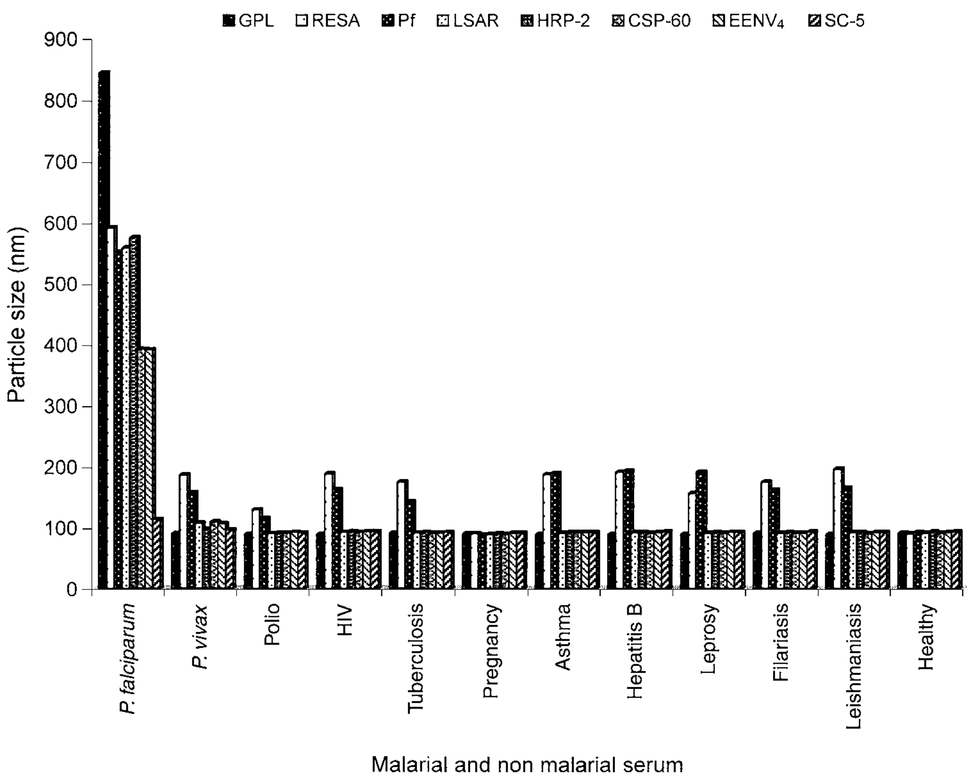

Fig. 4. Specificity of *P. falciparum* antigens and different serum reactivity by the LIA method.

particle size of polystyrene latex beads between *P. falciparum*-positive and healthy negative control serum. LIA is a basically antigen- and antibody-dependent direct binding assay with the formation of micro agglutination, and is perhaps slightly more sensitive than ELISA.

DISCUSSION

Several malaria antigens from different stages of the parasite cycle have been isolated and developed and have been used for antibody antigen reaction and assay studies. Glycosylphosphatidylinositols (GPIs) are a recently discovered class of glycolipids that anchor proteins and sugars into the plasma membrane in a wide range of organisms. Sherwood et al. (5) have identified and characterized the schizogony stage GPI and have shown that plasmodial glycolipid synthesis occurs concomitantly with glycoproteins synthesis. These schizont-synthesized glycolipids are associated with major changes in antigenicity, virulence, and the presence of surface antigens. Gerold et al. (8) have isolated a new inositol-based GPL lipid from merozoite stage *P. falciparum* and have shown that crude material extract induces tumor necrosis factor in the mouse model of malaria (18,19), suggesting that GPIs are new class of antigens responsible for cytokine-mediated pathology in this disease. They found that GPIs are the dominant agent of parasite origin and are responsible for the etiology of the cerebral syndrome in *P. falciparum* malaria. Understanding the role of glycolipids in *P. falciparum* may lead to new approaches for early detection and treatment of malaria. *P. falciparum* malaria antigen's behavior with antibodies under sero-diagnosis by immunological methods has been studied by several researchers (20-22). In the present study, the sensitivity and specificity aspect of antigens were examined in order to establish a successful diagnostic methodology. GPL1 and GPL2 were found to be very different in terms of their antigenic and antibody reactivity, which indicates that GPL1 is a parasite-origin product having a higher sensitivity and specificity than the nonparasite originated-GPL2 (Fig. 1). GPL2 appears to be of erythrocyte origin and without any antigenic properties (Fig. 2).

In this investigation, various malarial antigens were studied with nonmalarial disease antibodies to determine the specific nature of antigens for detection of disease by immunological methods. The specificity properties of *P. falciparum* antigens (GPL, RESA, *P. falciparum*, LSAR, HRP-2, CSP-60, $EENV_4$, and non-*P. falciparum* antigen (SC-5) were examined with sera from malarial and nonmalarial patients (Figs. 3 and 4). RESA, *P. falciparum*, LSAR, HRP-2, CSP-60, $EENV_4$, and SC-5 antigens showed approximately 10% cross-reactivity with HIV sera, but LSAR, HRP-2, CSP-60, $EENV_4$, and SC-5 showed 10-30% reactivity with hepatitis B serum by the ELISA method. RESA, LSAR, CSP-60, $EENV_4$ were found to show 10-20 % reactivity with HIV, leishmaniasis, and hepatitis B by the LIA method. However, 10-30% cross-reactivity with polio, HIV, tuberculosis, hepatitis B, leprosy, and filariasis in both methods was observed with SC-5 antigen. Nonspecific responses of antigens other than GPL1 could be due to antigenic variation. Specific serodiagnosis properties of antigens showed a differential pattern with malarial and nonmalarial diseases, with only GPL1 showing a very specific detection power. The difference in the specificity property may be due to the strong affinity of GPL1 antigen to *P. falciparum* antibodies. Oss and Walker (23), by means of a radioiodinated study, have shown that an Ag-Ab reaction occurs at a wide range of Ag-Ab ratios. GPL2 cannot distinguish between *P. vivax* and negative controls, but anti-GPL1 titers in *P. falciparum* sera are much higher than those in the *P. vivax* and negative control sera. These results indicate that GPL1 is of *P. falciparum* origin and is specific.

Antigenic properties of GPL1 antigen showed 96% ($P < 0.0001$) positivity for detection of disease by the LIA method, but the ELISA immunoassay showed 88% ($P < 0.0001$) positivity. The RESA antigen showed 78% serodiagnosis in LIA and 66% in ELISA. The *P. falciparum* antigen in LIA method showed 88% seropositivity, while in ELISA it was 70%. Decreased serodiagnosis responses were seen with LSAR, HRP-2, CSP-60, and $EENV_4$ antigens with LIA and ELISA methods, i.e., between 65-80% serodiagnosing capacity. SC-5 nonmalarial antigen showed a very low level of antigenic response, i.e., 10% sensitive ($P < 0.0001$) in the two methods. Many researchers have studied the sensitivity property of *P. falciparum* malaria patients with acridine orange (AO) staining, which can detect 52% of *P. vivax* infections and approximately 93% of *P. falciparum* infections (24-26). However, Cooke et al. (27), based on the benzothiocarboxypurine (BCP) method, have reported a sensitivity and specificity of 90% for detection of *P. falciparum* malaria. In the present study, GPL1 antigen showed 94-100% sensitivity and specificity for the detection of *P. falciparum* malaria under laboratory and field conditions.

The present results indicate that isolated GPL1 antigen purified from *P. falciparum* strain PSJ-M culture supernatant has very high immunosensitivity and specificity properties compared with other known antigens. In biochemical staining with orcinol, GPL supernatant has been found to be composed of sugar and lipid materials. Sherwood et al. (5) for the first time showed the role of cell-wall glycolipids synthesized by plasmodia and their association with antigenicity, virulence, and the development of surface antigens. Also, these plasmodial glycolipids were found to be composed of glucosamine, sugar, nucleotides, oligosaccharides, and proteolipids. Developmental studies of *P. falciparum* glycolipid antigens have showed the presence of glucosamine in the molecule (28-31), but our extracted and purified GPL1 from supernatant studied by Lowery et al. (13) and ninhydrin methods (42) showed the presence of no proteins or aminosugar (glucosamine). Earlier studies have also found that several protozoa synthesize protein free glycolipids, and that these glycolipids are major cellular glycoconjugates (32,33). Our study indicates that the isolated GPL is a protein-free malaria glycolipid.

The present study suggests that GPI plays an important role in toxicity versus an immunogenicity response, and that these antigens can be used in response to several clinical problems. Two commonly used parasite antigens for diagnosis of malaria (HRP-2 and parasite lactate-dehydrogenase [pLDH]) have been found to be very good antigens for detection of *P. falciparum* malaria (34-39). HRP-2- and pLDH-based assays sometimes give false-positive reactions in individuals who have recently been treated for malaria (40-42). However, GPL antigen developed and isolated from *P. falciparum* culture supernatant shows a similar level of detection and diagnosing capability under field and laboratory conditions, and we have not yet encountered any false positives.

ACKNOWLEDGMENTS

We would like to thank, Dr. Subba Rao, Director, Malaria Research Centre, for providing necessary laboratory facilities, and Mr. Surander Kumar, Mrs. Kamala Negi, and Mr. Ajit Singh for providing technical help.

REFERENCES

1. Makler, M. T., Palmer, C. J. and Ager, A. L. (1998): A review of practical techniques for the diagnosis of malaria. Ann. Trop. Med. Parasitol., 92, 419-433.
2. Contreras, C. E., Pance, A., Marcano, N., Gonazalez, N. and Bianco, N. (1999): Detection of specific antibodies to *Plasmodium falciparum* in blood bank donors from malarial-endemic and non-endemic areas of Venezula. Am J. Trop. Med. Hyg., 60, 948-953.
3. Ramasamy, R. and Reese, R. T. (1985): A role for carbohydrate moieties in the immune response to malaria. J. Immunol., 134, 1952-1955.
4. McDougal, J. B. and McDuffie, D. C. (1985): Immune complexes in man detection and clinical significance. Adv. Clin. Chem., 24, 1-60.
5. Sherwood, J. A., Spitalnik, S. L., Aley, S. B., Quaky, I. A. and Howard, R. J. (1986): *Plasmodium falciparum* and *P. knowiesi*: initial identification and characterization of malaria synthesized glycolipids. Exp. Parasitol., 62, 127-141.
6. Schofield, L., Vivas, L., Hackett, F., Gerold, P., Schwarz, R. T. and Tachado, S. (1993): Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-alpha-inducing toxin of *Plasmodium falciparum*: prospects for the immunotherapy of severe malaria. Ann. Trop. Med. Parasitol., 87, 617-626.
7. Clack, I. A. and Rockett, K. A. (1994): The cytokine theory of human cerebral malaria. Parasitol. Today, 10, 410-412.
8. Gerold, P., Schofielf, L., Blackman, M. J., Holder, A. A. and Schwarz, R. T. (1996): Structural analysis of the glycoxyl phosphatidylinositol membrane anchor of the merozoite surface proteins 1 and 2 of *Plasmodium falciparum*. Mol. Biochem. Parasitol., 75, 131-143.
9. Nezlin, R. (2000): A quantitative approach to the determination of antigen in immune complexes. J. Immunol. Method, 237, 1-17.
10. Trager, W. and Jensen, J. B. (1976): Human malarial parasites in continuous culture. Science. 193, 673-675.
11. Folch, J., Lees, M. and Stanley, G. H. S. (1957): A simple method for the isolation and purification of total lipid from animal tissues. J. Biol. Chem., 226, 497-509.
12. Dubon, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956): Colorimetric method for determination of sugars and related substances. Anal. Chem., 28, 350-356.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol.Chem., 193, 265-275.
14. Bartlett, G. R. (1959): Phosphorus assay in column chromatography. J. Biol. Chem., 234, 466- 468.
15. Kundu, S. K. (1981): Thin layer chromatography of neutral glycosphingolipids and gangliosides. Methods Enzymol., 72, 185-204.
16. Roy, A., Biswas, S., Kabilan, L. and Sharma, V. P. (1995): Application of simple peptide ELISA for stratification of malaria endemicity. Indian J. Malarilogy, 32, 164-173.
17. Bhakat, P., Roy, A. and Roy, K. B. (1999): Laser light scattering immunoassay for malaria. J. Immunoassay, 20, 103-144.
18. Hotez, P. J., Le Trang, N., Fairlamb, A. H. and Cerami, A. (1984): Lipoprotein lipase suppression in 3T3-LI cells by a haematoprotozoan-induced mediator from peritoneal exudates cells. Parasitol. Immunol., 6, 203-209.
19. Bate, C. A., Taverne, J. and Playfair, J. H. (1992): Detoxified exoantigens and phosphatidylinositol derivatives inhibit tumor necrosis factor induction by malarial exoantigens. Infect. Immun., 60, 1894-1901.
20. Brenier-Pinchart, M. P., Pinel, C., Croisonnier, A., Brion, J. P., Faure, O., Ponard, D. and Ambroise-Thomas, P. (2000): Diagnosis of malaria in non-endemic countries by the Parasight-F® Test. Am. J. Trop. Med. Hyg., 63, 150-152.
21. Okech, B. A., Nalunkuma, A., Okello, D., Pang, X., Suzue, K., Li, J., Horii, T. and Egwang, T. G. (2001): Natural human immunoglobulin G subclass responses to *Plasmodium falciparum* serine repeat antigen in Uganda. Am. J. Trop. Med. Hyg., 65, 912-917.
22. Mya, M. M., Saxena, R. K and Roy, A. (2002): Sensitivity and specificity of isolated antigen from *Plasmodium falciparum* culture supernatant. Indian J. Clin. Biochem., 17, 75-82.
23. van Oss, C. J. and Walker, J. (1987): Concentration dependence of the binding constant of antibodies. Mol. Immunol., 24, 715-717.
24. Delacollette, D. and Stuyft, V. P. (1994): Direct acridine orange staining is no a 'miracle' solution to the problems of malaria diagnosis in the field. Tran. Roy. Soc. Trop. Med. Hyg., 88, 187-188.
25. Clendennen, T. E., Long, G. W. and Baird, J. K. (1995): QBC and Giemsa stained thick blood films diagnostic performance of laboratory technologists. Tran. Roy. Soc. Trop. Med. Hyg., 89, 183-184.
26. Lowe, B. S., Jeffa, N. F., New, L., Pedersen, C., Engbae, K. and Marsh, K. (1996): Acridine orange fluorescence technique as alternatives to traditional Giemsa staining for the diagnosis of malaria in developing countries. Trans. Roy. Soc. Trop. Med. Hyg., 90, 34-36.
27. Cooke, A. H., Morris-Jones, S., Horton, J., Greenwood, B., Moody, A.H. and Chiodini, P. L. (1993): Evaluation of benzothiocarboxypurine for malaria diagnosis in an endemic area. Tran. Roy. Soc. Trop. Med. Hyg., 87, 549.
28. Siddqui, W. A., Schnell, J. V. and Richmond, C. S. (1974): In vitro *Plasmodium falciparum* at high parasitemia. Am. J. Trop. Med. Hyg., 23, 1015-1018.
29. Halder, K., Ferguson, M. A. J., George, A. and Cross, A. M. (1985): Acylation of a *Plasmodium falciparum* merozoite surface antigens sn-1, 2-diacyl Glycerol. J. Biol. Chem., 260, 4969-4974.
30. Halder, K., Henderson, C. L., George, A. and Cross, A. M. (1986): Identification of the parasite transferring receptor of *Plasmodium falciparum*-infected erythrocytes and its acylation via 1, 2-diacyl-sn-glycerol. Proc. Natl. Acad. Sci. USA, 83, 8565-8569.
31. Smythe, J. A., Coppel, R. L., Brown, G. V., Ramasamy, R., Kemp, D. J. and Anders, R. F. (1988): Identification of two integral membrane proteins of *Plasmodium falciparum*. Proc. Natl. Acad Sci.USA, 85, 5195-5199.
32. McConville, M. J. and Blackwell, J. M. (1991): Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. J. Biol. Chem., 266, 15170-15179.
33. Turco, S. J. and Descoteaux, A. (1992): The lipophosphoglycan of Leishmania parasites. Ann. Rev. Microbiol., 46, 65-94.

34. Howard, R. J., Uni, S., Alkawa, M., Aley, S., Leech, J. H., Wellems, T. E., Renes, J. and Taylor, D. W. (1986): Secretion of a malaria histiden rich protein (PfHRP-II) from *Plasmodium falciparum* infected erythrocytes. J. Cell. Biol., 103, 1269-1277.
35. Rock, E. P., Marsh, K., Saul, S. J. and Wellems, R. J. (1987): Comparative analysis of the *Plasmodium histidine* rich proteins HRP-1, HRP-2 and HRP-3 in malaria diagnosis of diverse origin. Parasitology, 95, 209-227.
36. Oduola, A. M. J., Omitowoju, G. O., Sowunmi, A., Makler, M. T., Falade, C. O., Kyle, D. E., Fehinto, La F. A., Ogundahunsi, A. O. T., Schuster, B. G. and Milhous, W. K. (1987): *Plasmodium falciparum*: evaluation of lactate dehydrogenase in monitoring therapeutic response to standard antimalarial drugs in Nigeria. Exp. Parasitol. 87, 283-289.
37. Dietze, R., Perkins, M., Boulos, M., Luz, F., Reller, B. and Corey, C. R. (1995): The diagnosis of *Plasmodium falciparum* infection using a new antigen detection system. Am. J. Trop. Med. Hyg., 52, 45-49.
38. Palmer, C. J., Lindo, J. F., Klaskala, W., Quesada, J., Kaminaky, R., Baum, M. K. and Ager, A. L. (1998): Evaluation of the OpitiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. J. Clin. Microbiol. 36, 203-206.
39. Beadle, C., McElroy, P. D., Oster, C. N., Beier, J. C., Oloo, A. J., Onyango, F. K., Chumo, D. K., Bales, J. D., Sherwood, J. A. and Hoffman, S. L. (1995): Impact of transmission intensity and age on *Plasmodium falciparum* density and associated fever: implications for malaria vaccine design. J. Infect. Dis., 172, 1047-1054.
40. Schiff, C. J., Premji, Z. and Minjas, J. N. (1993): The rapid manual ParaSight®-F test. A new diagnostic tool for *Plasmodium falciparum* infection. Trans. Roy. Soc. Trop. Med. Hyg., 87, 646-684.
41. Schiff C. J., Minjas, J. N. and Premji, Z. (1994): The ParaSight-F test: a simple rapid manual dipstick test to detect *Plasmodium falciparum* infection. Parasitol. Today, 10, 494-495.
42. Moore, S. J. (1968): Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem., 243, 6281-6283.