Original Article

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

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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, EENV4, 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, and 155 origin). Synthetic antigens have been determined and compared 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 sera 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, EENV4) 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
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: (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 correlator, 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, glycoprophatidylinositol 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 malarigenic 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 µg/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 antibody binding showed that at 1:100 antigen dilutions, GPL1 had one OD with the *P. falciparum*-positive serum while less than 0.4 OD for the *P. vivax* and control serum.

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

<table>
<thead>
<tr>
<th>Method</th>
<th>Content</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin-Layer Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodine vapor</td>
<td>Glycolipid</td>
<td>Present</td>
</tr>
<tr>
<td>Bial's reagent (Sigma: O-7875)</td>
<td>Sugars</td>
<td>Present</td>
</tr>
<tr>
<td>Zinzadze reagent test (Sigma No. M-3389)</td>
<td>Phospholipid</td>
<td>Present</td>
</tr>
<tr>
<td>Barlett method</td>
<td>Phosphate</td>
<td>Present</td>
</tr>
<tr>
<td>2% Ninhydrin method</td>
<td>Aminosugar</td>
<td>Absent</td>
</tr>
<tr>
<td>High-Performance Thin-Layer Chromatography</td>
<td>Glycoporphospholipid</td>
<td>Present</td>
</tr>
<tr>
<td>Chemical Tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowry method</td>
<td>Protein</td>
<td>0.035%</td>
</tr>
<tr>
<td>5% Phenol-H2SO4 test</td>
<td>Total Sugars (Carbohydrate)</td>
<td>0.25%</td>
</tr>
<tr>
<td>Gas-Liquid Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>0.35 cm²</td>
<td>Mean</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.35 cm²</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>0.25 cm²</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.25 cm²</td>
<td></td>
</tr>
</tbody>
</table>

[Estimation of sugar in GPL1: A sensitive area planimeter (cm²) was used for the measurement of the area of sugar moieties in the graph. Ratio: (Xylose:3, Mannose:3, Galactose:1.5, Glucose:1.5). Total sugar = 0.25%]
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>
<thead>
<tr>
<th>S. No.</th>
<th>Particulars (( n=50 ))</th>
<th>GPL1</th>
<th>RESA</th>
<th>Pf</th>
<th>LSAR</th>
<th>HRP-2</th>
<th>CSP-60</th>
<th>EENV4</th>
<th>SC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIA (nm)</td>
<td>ELISA (OD)</td>
<td>LIA (nm)</td>
<td>ELISA (OD)</td>
<td>LIA (nm)</td>
<td>ELISA (OD)</td>
<td>LIA (nm)</td>
<td>ELISA (OD)</td>
<td>LIA (nm)</td>
</tr>
<tr>
<td>1</td>
<td>Mean</td>
<td>804.96</td>
<td>0.79</td>
<td>551.51</td>
<td>0.56</td>
<td>492.54</td>
<td>0.54</td>
<td>461.88</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>182.23</td>
<td>0.13</td>
<td>272.64</td>
<td>0.21</td>
<td>223.29</td>
<td>0.21</td>
<td>218.57</td>
<td>0.12</td>
</tr>
<tr>
<td>+ve subjects</td>
<td>48</td>
<td>44</td>
<td>39</td>
<td>33</td>
<td>44</td>
<td>35</td>
<td>39</td>
<td>34</td>
<td>43</td>
</tr>
<tr>
<td>(% +ve)</td>
<td>96%</td>
<td>88%</td>
<td>78%</td>
<td>66%</td>
<td>88%</td>
<td>70%</td>
<td>78%</td>
<td>68%</td>
<td>86%</td>
</tr>
<tr>
<td>Control</td>
<td>Mean</td>
<td>98.36</td>
<td>0.20</td>
<td>98.72</td>
<td>0.15</td>
<td>97.13</td>
<td>0.16</td>
<td>97.71</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>3.85</td>
<td>0.02</td>
<td>4.89</td>
<td>0.06</td>
<td>3.45</td>
<td>0.05</td>
<td>4.28</td>
<td>0.05</td>
</tr>
<tr>
<td>False +ve</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>0%</td>
<td>0%</td>
<td>2%</td>
<td>8%</td>
<td>2%</td>
<td>6%</td>
<td>4%</td>
<td>6%</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>Cut of value (mean ± 2SD)</td>
<td>106.07</td>
<td>0.24</td>
<td>108.5</td>
<td>0.26</td>
<td>93.98</td>
<td>0.25</td>
<td>107.77</td>
<td>0.29</td>
<td>105.69</td>
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<tr>
<td>P value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

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; EENV4=Tetramer from RESA/Pf155; SC-5=Non malarial peptide.

![Fig. 1. Study of GPL1 malaria culture supernatant by a Hewlett Packard Glass capillary gas chromatogram (ECNSS 3%).](image1.png)

Fig. 1. Study of GPL1 malaria culture supernatant by a Hewlett Packard Glass capillary gas chromatogram (ECNSS 3%).
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
Several malaria antigens from different stages of the parasite cycle have been isolated and developed and have been used for antigen reaction and assay studies. Glycosylphosphatidylinositol (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 plasmoidal 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, EENV4, 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, EENV4, and SC-5 antigens showed approximately 10% cross-reactivity with HIV sera, but LSAR, HRP-2, CSP-60, EENV4, and SC-5 showed 10-30% reactivity with hepatitis B serum by the ELISA method. RESA, LSAR, CSP-60, EENV4 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 titer 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 EENV4 antigens with LIA and ELISA methods, i.e., between 65-80% serodiagnostic 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 specificity 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 plasmoidal glycolipids were found to be composed of glucosamine, sugar, nucleotides, oligosaccharides, and proteolipids. Developmental studies of P. falciparum glycolipid antigens have shown 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 GPL 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.

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REFERENCES


