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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
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Señor

Jefe de la División de Nuevas Creaciones

Superintendencia de Industria y Comercio

Ministerio de Comercio, Desarrollo, Industria y Turismo

E. S. D.

Ref.: Expediente No. **01.011.966**

Solicitud de Patente de Invención para:

**"INMUNOENSAYO Y REACTIVO DIAGNÓSTICO PARA LA DETECCIÓN DE
LA MALARIA".**

Solicitante: **LG CHEMICAL LTD.**

RESPUESTA AL EXAMEN DE BUSQUEDA DE ANTERIORIDADES

MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, en atención a su oficio No. **10443** Notificado el **14 de Septiembre de 2006**, atentamente me permito dar repuesta al mismo de la siguiente manera:

Establece el funcionario examinador en forma resumida que *"como resultado de la búsqueda de anterioridades se encontró que la solicitud en estudio corresponde con la solicitud europea y publicada con el número WO01/61032. El análisis del informe de búsqueda internacional de la solicitud extranjera establece como documentos que afecta la novedad del objeto de la solicitud Colombiana los siguientes:*

1. **SOARES I S ET AL:** "Longevity of naturally acquired antibody responses to the N- and C-Terminal regions of plasmodium vivax merozoite surface protein 1" American Journal of Tropical Medicine & Hygiene, Lawrence, KS, US vol 60, no.3, 1999, pages 357-363, XP009028883 ISSN: 0002-9637.
2. **TYAGI P ET AL:** "Naturally occurring plasmodia-specific circulating immune complexes in individuals of Malaria endemic areas in India" INDIAN JOURNAL

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- OF MALARIOLOGY, MALARIA RESEARCH CENTER, DEHLI, IN, Vol 36, no 1 / 2, March 1999. (1999-03), pages 12-18, XP009028923.
3. **RAY K ET AL:** "Evaluation of indirect immunofluorescent antibody test for detection of IGM specific antibodies in Malaria" JOURNAL OF COMMUNICABLE DISEASES, INDIA SOCIETY FOR MALARIA AND OTHER COMMUNICABLE DISEASES, DEL, IN, vol 24, no.2, June 1992 (1992-06), pages 82-86, XP009028938 ISSN:0019-5138.
 4. **GIBSON H L ET AL:** "Structure and expression of the gene for PV200 a major blood-stage surface antigen of plasmodium-vivax" Molecular and Biochemical Parasitology, vol 50, no.2, 1992, pages 325-334, XP000199300 ISSN: 0166-6851.
 5. **GENTZ R ET AL:** "Major Surface antigen P190 of Plasmodium Falciparum: Detection of common epitopes present in a variety of plasmodia isolates" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol, 7 no 1, 1988 pages 225-230, XP002057622 ISSN: 0261-4189.
 6. **SOARES ET AL:** "Acquired immune responses to the N- and C-terminal regions of plasmodium vivax merozoite surface protein 1 in individuals exposed to malaria "INFECT. IMMUN.vol 65, 1997, pages 1606-1614, XP002972558.
 7. **PAN ET AL:** "Vaccine candidate MSP-1 from plasmodium falciparum: a redesigned 4917 bp polynucleotide enables synthesis and isolation of full-length protein from Escherichia coli and mammalian cells "NUCLEIC ACIDS RES. Vol 27, 15 February 1999, pages 1094-1103, XP000953100
 8. **FRUH ET AL:** "A new tool for the serodiagnosis of acute plasmodium falciparum malaria in individuals with primary infection" J. IMMUNOL METHODS vol.122, 15 August 1989, pages 25-32, XP002972559.
 9. **SANCHEZ ET AL:** "Antibody response to plasmodium vivax antigens in human malaria" AM. J. TROP.MED. HYG. Vol. 50, March 1994, pages 329-338, XP002973046.
 10. **KASLOW ET AL:** "Expression and immunogenicity of the C-terminus of a major blood-stage surface protein of plasmodium vivax, Pv200(19) secreted from saccharomyces cerevisiae "IMMUNOL. LETT. Vol. 51, july 1996, pages 187-189, XP002972560
 11. **DELCARDAYRE ET AL:** "Engineering ribonucleas A production, purification and characterization of wild-type enzyme and mutants at G1n11" PROTEIN ENG. Vol.8, 1995, pages 261-273, XP002972561.

"Dado que fue imposible la consecución de los artículos citados anteriormente, se requiere la presentación de los mismos".

Con el objeto de atender a la anterior observación, me permito presentar copia completa de cada uno de los documentos arriba citados.

Los argumentos para demostrar que estos artículos no afectan los requisitos de patentabilidad de la presente invención, se presentan en la respuesta al examen de patentabilidad.

Cumplido así con lo dispuesto, atentamente solicito a Ud. se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

Del Señor Jefe,

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Margarita Castellanos Abondano

C.C. No. 39.779.654 de Usaquén

T.P.A. No. 70819 del C. Sup. de la Judicatura.

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LONGEVITY OF NATURALLY ACQUIRED ANTIBODY RESPONSES TO THE N- AND C-TERMINAL REGIONS OF *PLASMODIUM VIVAX* MEROZOITE SURFACE PROTEIN 1

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Abstract. In an earlier study, we found that individuals with patent infection had significantly higher IgG antibody titers to the 19-kD C-terminal region of *Plasmodium vivax* merozoite surface protein 1 (PvMSP1) than individuals treated for malaria 1-4 months earlier. These results suggested that the antibody levels decreased rapidly following treatment. The present study was designed to determine the persistence of antibody response to the N- and C-terminal regions of PvMSP1 after infection with *P. vivax* in individuals from the city of Belém in northern Brazil. Our results demonstrated that the vast majority of individuals had a significant decrease in antibody titers to the C-terminal region of PvMSP1 in a period of two months following treatment. Among responders to the C-terminal region, 44.4% became serologically negative and 44.4% had their antibody titers reduced by an average of 13-fold. Only 11.2% of the individuals had their antibody titers maintained or slightly increased during that period. A decrease in the antibody response to the recombinant protein representing the N-terminal region of PvMSP1 was also noted; however, it was not as dramatic. The rapid decrease in the antibody levels to the C-terminal region of PvMSP1 might contribute to the high risk of reinfection in these individuals.

Plasmodium vivax is the second most prevalent species that causes malaria in humans and accounts for approximately 35 millions cases of the disease every year.¹ In many countries, e.g., Brazil, *P. vivax* is the most common species, where it caused 76.8% of the 405,051 cases reported in 1997.²

In spite of being highly prevalent in many parts of the world, the immunologic mechanisms operating in individuals exposed to *P. vivax* are poorly understood. We have characterized serum antibody and T cell reactivity of individuals from northern Brazil recently exposed to *P. vivax* malaria with 11 recombinant proteins representing the N- and C-terminal regions of the merozoite surface protein 1 of *P. vivax* (PvMSP1).³ We found that a high frequency of individuals had IgG antibodies and T cell reactivity to at least one recombinant protein derived from PvMSP1.³ The recombinant protein, which is based on the 19-kD C-terminal region of PvMSP1 (PvMSP1_C) containing the two epidermal growth factor (EGF)-like regions, was the most immunogenic during natural infection in humans. Antibodies or T cells of 83.8% of the individuals recognized this recombinant protein.³ Furthermore, the antibody titers to the C-terminal region of PvMSP1 were higher than the titers to the N-terminal region. This high frequency of responders was also described in an independent survey performed in Papua New Guinea, where the sera of more than 80% of the individuals reacted with a recombinant protein representing PvMSP1.⁴ These immunoepidemiologic studies on naturally acquired immunity to the C-terminal of PvMSP1 are of particular importance since this region of MSP1 is being intensively studied as a candidate for development of a vaccine against malaria.^{1,3-15}

We also found in our study that individuals with patent infection had significantly higher IgG antibody titers to the C-terminal region of PvMSP1 than individuals treated for malaria 1-4 months before, suggesting that the antibody levels decreased rapidly after treatment. Based on this finding,

the present study was designed to determine the persistence of antibody levels to the N- and C-terminal regions of PvMSP1 after infection with *P. vivax* in individuals from the city of Belém in the northern Brazil.

SUBJECTS, MATERIALS, AND METHODS

Subjects. As described in an earlier study,³ most (66.3%) of the residents of the city of Belém, state of Pará in northern Brazil are life-long residents and they were exposed to *P. vivax*-infected mosquitoes during short-term stays in regions surrounding the city where transmission occurs. Only cases of *P. vivax* are reported in these areas and low levels of transmission are observed throughout the year. At the end of the rainy season (June through August), a slightly higher number of cases are observed. These individuals reported to the Instituto Evandro Chagas (Belém, Pará, Brazil) for diagnosis and treatment. This made it possible to estimate precisely the number of malaria episodes.

This study was approved by the Committee of Ethics of the Federal University of São Paulo-Escola Paulista de Medicina. After verbal consent was provided, 5 ml of venous blood was collected from 99 individuals in non-heparinized tubes and used as a source of serum. At the time their blood samples were collected, they had patent *P. vivax* infections as determined by microscopic analysis of Giemsa-stained blood drops before treatment. These individuals initiated treatment for *P. vivax* malaria the same day the blood samples were collected. The standard treatment consisted of one oral dose of 600 mg of chloroquine and 30 mg of primaquine administered the day the diagnosis was made. These patients received daily doses of 30 mg of primaquine over the next six days. The mean $\pm$ SD age of this group was 25.2 ± 11.9 years and 70% were males. Blood samples of 36 individuals from this group were collected two months after treatment. At that time, these individuals did not present with patent *P. vivax* infections. The mean $\pm$ SD age of these subjects was

25.6 $\pm$ 13.3 years and 61.1% were males. It was not possible to evaluate the antibody response of a larger group of individuals or after that period since once cured, most of them refuse to donate blood samples a second time.

Recombinant PvMSP1 proteins. The N- and C-terminal regions of PvMSP1 from the Belém strain of *P. vivax* were expressed as glutathione S-transferase (GST) fusion proteins. The detailed construction of these fusion proteins has been described elsewhere.^{3,16} The recombinant protein ICB2-5 contains 506 amino acids located at the N-terminal region of PvMSP1. Protein PvMSP1₁₉ encodes 111 amino acids and contains the two EGF-like motifs described for the C-terminal region of other MSP1 molecules.¹⁷ As a control, GST was produced alone. Recombinant proteins and GST were affinity purified on glutathione-Sepharose 4B columns (Pharmacia, Uppsala, Sweden), their purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein concentration was measured by the Bio-Rad (Hercules, CA) protein assay.

Immunoassays. Detection of antigen specific IgG antibodies by ELISA. Sera from 99 individuals were tested for reactivity with the PvMSP1 recombinant proteins by an ELISA. Microtiter plates (Costar, Cambridge, MA) were coated with 200 ng/well of affinity-purified fusion proteins or GST and incubated overnight at 4°C and washed three times with phosphate-buffered saline (PBS) plus 0.05% Tween-20. The plates were then blocked at 37°C for 2 hr with 5% nonfat milk in PBS. In the first test, serum samples were added to duplicate wells at dilution of 1:100. The sera that were positive for recombinant proteins ICB2-5 or PvMSP1₁₉ were titrated with subsequent two-fold serial dilutions up to 1:102,400. After incubation for 2 hr at room temperature, unbound material was washed away with PBS-0.05% Tween, and peroxidase-conjugated goat anti-human IgG (Fc specific) (Sigma, St. Louis, MO) diluted 1:10,000 was added to each well. In some experiments we used peroxidase-conjugated goat anti-human IgM (Sigma) diluted 1:4,000. After a 1-hr incubation at room temperature, excess-labeled antibody was removed by washing with PBS-0.05% Tween, and the reaction was developed with *o*-phenylenediamine (Sigma). Plates were read at 492 nm on an ELISA reader (Multiskan MS; Labsystems, Helsinki, Finland). All optical density values at 492 nm (OD₄₉₂) represent binding of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. Each serum was tested in duplicate and the OD₄₉₂ values were averaged. Cut-off points were set at 3 SD above the mean OD₄₉₂ value of sera from 30 healthy individuals from the city of São Paulo who were never exposed to malaria.

Enzyme-linked immunosorbent assay to identify antigen-specific antibodies of distinct IgG subclass. This ELISA was performed as described earlier except that subclass-specific, mouse anti-human IgG monoclonal antibodies (MAbs) were used as the second-step reagent. These MAbs recognize human IgG1, IgG2, IgG3, or IgG4 (Sigma) and were diluted 1:8,000 in PBS-5% nonfat milk. After incubation for 1 hr at room temperature, the plates were washed with PBS-0.05% Tween and peroxidase-labeled anti-mouse IgG heavy and light chain (Kirkgaard and Perry, Gaithersburg, MD) was added at a final dilution of 1:4,000. The plates were washed with PBS-0.05% Tween and developed as described earlier.

The OD₄₉₂ values represent binding of each subclass of IgG to the recombinant protein after subtraction of binding of the same serum to GST alone. Only serum samples that had IgG specific for recombinant proteins ICB2-5 or PvMSP1₁₉ were tested in this assay. Each serum was tested in duplicate and the OD₄₉₂ values were averaged. Cut-off points were set at 3 SD above the mean OD₄₉₂ value of sera from 10 healthy individuals from the city of São Paulo who were never exposed to malaria.

Statistical analysis. Kruskal-Wallis one-way analysis of variance, McNemar comparison of proportions, and a chi-square test were performed using the True Epistat software package (Dr. Tracy L. Gustafson, Richardson, TX).

RESULTS

In earlier studies, we observed that the IgG antibody titers to the C-terminal region of PvMSP1 were higher in serum samples from individuals with patent infections than in the sera from individuals treated for malaria 1-4 months earlier.³ To determine precisely whether there was a significant decrease in antibody titers after treatment, the sera of 99 individuals were collected during patent infection. Two months after treatment, we obtained blood samples from 36 of these individuals. Their IgG antibody titers were determined by ELISA using PvMSP1₁₉ antigen, which contained the C-terminal 111 amino acids representing the two EGF-like regions of PvMSP1. We also used a recombinant protein containing amino acids 170-675 of the N-terminal region of PvMSP1 (Belém strain) (ICB2-5) to compare the immune response to the N- and C-terminal regions of PvMSP1.

As shown in Figure 1, the proportions of individuals with patent infections who recognize proteins ICB2-5 or PvMSP1₁₉ were 63.8% and 75%, respectively. After treatment, there was a significant reduction in the frequency of responders. The proportions of responders to proteins ICB2-5 and PvMSP1₁₉ were reduced to 38.9% and 47.2%, respectively, demonstrating that some of the individuals became serologically negative in a period of two months after contact with the parasite ($P = 0.0175$ and $P = 0.0065$ to ICB2-5 and PvMSP1₁₉, respectively, by McNemar comparison of proportions). When sera from individuals who recognized each recombinant protein were titrated, we observed that in individuals with patent infections, antibody titers to proteins ICB2-5 and PvMSP1₁₉ were significantly higher than antibody titers of serum samples collected after treatment. While the decrease in antibody titers to protein ICB2-5 was barely significant ($P = 0.038$, by Kruskal-Wallis one-way analysis of variance), the reduction in antibody titers to PvMSP1₁₉ was very dramatic ($P = 0.0058$). These results also confirmed our earlier observation that IgG antibody titers to PvMSP1₁₉ were higher than titers to protein ICB2-5 in individuals exposed to malaria.³

Among individuals who responded to protein ICB2-5, 43.5% became negative in a period of two months following treatment. Their antibody titers decreased by more than 13.6-fold (Table 1, Group 1). A total of 39.1% of the individuals had mean $\pm$ SD decreases in their antibody titers of 11.3 $\pm$ 6.6-fold (Group 2). Only 17.4% of individuals had their antibody titers maintained or increased during that period (Group 3). However, it is noteworthy that these individuals

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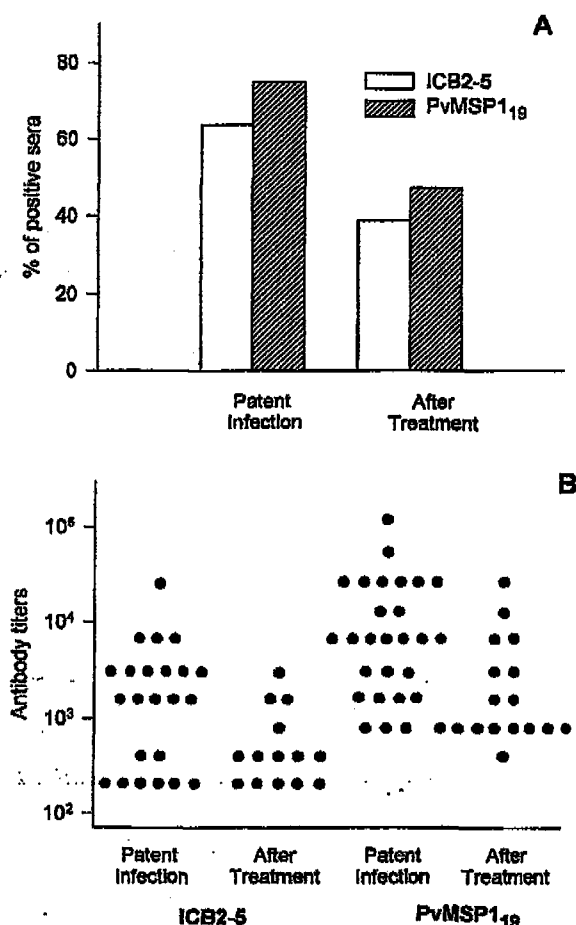


FIGURE 1. Antibody response to recombinant proteins ICB2-5 or PvMSP1₁₉ during patent *Plasmodium vivax* infection or after treatment. A, percentage of responders was estimated from 36 individuals and calculated as those serum samples that showed optical density at 492 nm (OD₄₉₂) values 3 SD deviations above the mean OD₄₉₂ obtained from serum samples of 30 healthy individuals who were never exposed to malaria. Serum samples were tested at a dilution of 1:100. The cut-off values for each protein were ICB2-5, 0.250 and PvMSP1₁₉, 0.200. The frequency of positive sera during patent infection was higher than the frequency after treatment ($P = 0.0175$ and $P = 0.0065$, for proteins ICB2-5 and PvMSP1₁₉, respectively, by McNemar comparison of proportions). B, antibody titers of each individual represent the last dilution that had an OD₄₉₂ higher than 0.1 OD/ml after subtraction of binding of the same serum antibodies to glutathione-S-transferase alone. The results of statistical analysis determined that during patent infection, antibody titers to proteins ICB2-5 and PvMSP1₁₉ were higher than after treatment ($P = 0.038$ and $P = 0.0058$ for ICB2-5 and PvMSP1₁₉, respectively, by Kruskal-Wallis one-way analysis of variance).

had relatively low antibody titers ranging from 1:200 to 1:400.

As shown in Table 2, among individuals who responded to PvMSP1₁₉, 44.4% became serologically negative with a reduction in antibody titers ranging from 16- to 512-fold (Group 4). Other individuals (44.4%) had their antibody titers reduced by a mean $\pm$ SD of 13.0 $\pm$ 10.0-fold (Group

TABLE 1
Variation in antibody titers to protein ICB2-5*

Number of individuals	Antibody titers during patient infection	Antibody titers 2 months after treatment	Decrease in antibody titer (fold)
Group 1			
2	1:3,200	Neg. (<1:100)	>32
4	1:1,600	Neg. (<1:100)	>16
4	1:200	Neg. (<1:100)	>2
Group 2			
1	1:25,600	1:1,600	16
1	1:6,400	1:3,200	2
2	1:6,400	1:400	16
1	1:3,200	1:1,600	2
3	1:3,200	1:200	16
1	1:1,600	1:800	2
Mean $\pm$ SD reduction = 11.3 $\pm$ 6.6			
Group 3			
2	1:400	1:400	0
1	1:200	1:200	0
1	1:200	1:400	2 (increase)
1	Neg. (<1:100)	1:200	(increase)

* Neg. = negative.

5). Antibody titers during this period were maintained or increased in only 11.2% of the individuals (Group 6). Their antibody titers ranged from 1:1,600 to 1:25,200.

We subsequently evaluated whether there was a decrease in specific subclasses of IgG. In sera collected from individuals with patent infections, IgG1 and IgG3 were the predominant subclasses that recognized protein ICB2-5 (Figure 2A). Only 8.7% or 14% of the individuals had IgG2 or IgG4 antibodies specific for this polypeptide, respectively. We then compared the frequency of responders during patent infection and after treatment and found that a significant ($\geq$

TABLE 2
Variation in antibody titers to protein PvMSP1₁₉*

Number of individuals	Antibody titers during patient infection	Antibody titers 2 months after treatment	Decrease in antibody titer (fold)
Group 4			
1	1:51,200	Neg. (<1:100)	>512
2	1:25,600	Neg. (<1:100)	>256
2	1:6,400	Neg. (<1:100)	>64
4	1:1,600	Neg. (<1:100)	>16
3	1:800	Neg. (<1:100)	>8
Group 5			
1	1:102,400	1:6,400	16
1	1:25,600	1:1,600	16
2	1:25,600	1:800	32
2	1:12,800	1:800	16
2	1:6,400	1:3,200	2
1	1:6,400	1:800	8
2	1:3,200	1:800	4
1	1:3,200	1:400	8
Mean $\pm$ SD reduction = 13.0 $\pm$ 10			
Group 6			
1	1:25,600	1:25,600	0
1	1:12,800	1:25,600	2 (increase)
1	1:6,400	1:6,400	0
1	Neg. (<1:100)	1:1,600	(increase)
1	Neg. (<1:100)	1:1,600	(increase)

* Neg. = negative.

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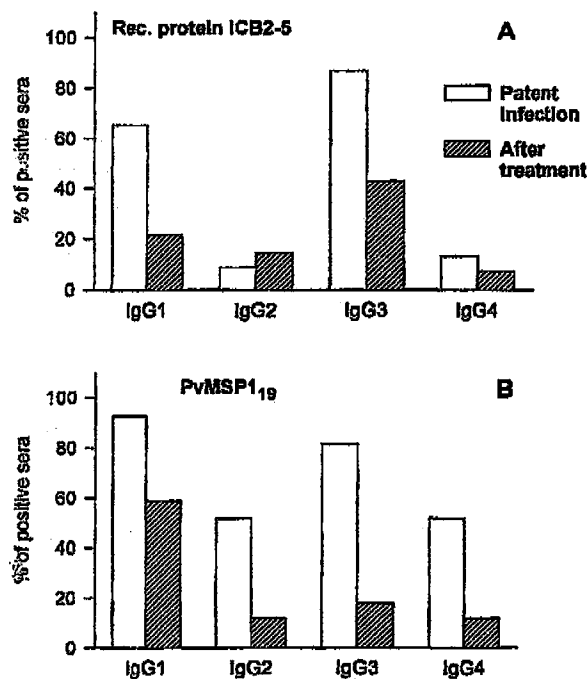


FIGURE 2. Frequency of individuals with antibodies of distinct subclass of IgG during patent infection and after treatment. The ELISA was performed using subclass-specific, anti-human IgG1, IgG2, IgG3, or IgG4 mouse monoclonal antibodies. Only serum samples that had total IgG specific for proteins ICB2-5 or PvMSP1₁₉ were tested in this assay. The percentage of responders was calculated as those serum samples that presented optical density at 492 nm (OD₄₉₂) values 3 SD above the mean OD₄₉₂ obtained from serum samples of 10 healthy individuals who were never exposed to malaria. Serum samples were tested at a dilution of 1:1 00. The OD₄₉₂ values represent binding of IgG to the recombinant (Rec.) protein after subtraction of binding of the same serum to glutathione-S-transferase alone. The cut-off values for each recombinant protein were 0.007, 0.960, 0.012, and 0.10 for ICB2-5 IgG1, IgG2, IgG3, and IgG4, respectively, and 0.074, 0.503, 0.037, and 0.100 for PvMSP1₁₉ IgG1, IgG2, IgG3, and IgG4, respectively. A, ICB2-5. The frequency of responders with IgG1 or IgG3 was higher during the infection than after treatment ($P < 0.001$ in both cases, by McNemar comparison of proportions). B, PvMSP1₁₉. The frequency of responders with IgG1, IgG2, IgG3, or IgG4 was higher during the infection than after treatment ($P < 0.001$ in all cases).

50%) proportion of individuals became negative after treatment. The frequency of individuals with specific IgG1 or IgG3 decreased significantly ($P < 0.001$). In contrast, the frequency of individuals with IgG2 or IgG4 did not change.

When the same type of analysis was performed with sera from individuals who responded to PvMSP1₁₉, we found that IgG1 and IgG3 were also the predominant subclasses of antibodies during patent infection (Figure 2B). However, significant proportion of individuals also had IgG2 or IgG4 antibodies. The comparison of the proportion of responders during patent infection and after treatment showed that all frequencies obtained from the second group were lower ($P < 0.001$). In the case of IgG1, we observed that the frequency of responders did not decrease as much as with the other subclasses.

The rapid decrease in IgG antibody response to recombinant proteins of PvMSP1 could be explained if these individuals had an impaired secondary antibody response during the second or third episodes of malaria. This hypothesis could be tentatively tested by two approaches. First, we determined the frequency of sera that had specific IgM antibodies in individuals in their first, second, or third episode of the disease. Later, we studied whether there was a boosting effect that would generate a higher antibody titer during the second or third *P. vivax* infection. For that purpose, we divided the sera of the 99 individuals according to the number of malaria episodes and determined the frequency of sera containing specific IgM or IgG antibodies. We also determined the titers of IgG antibodies specific for recombinant proteins ICB2-5 and PvMSP1₁₉.

As shown in Figure 3, we found that the frequency of serum samples containing IgM specific for PvMSP1₁₉ was higher in individuals during their first malaria episode when compared with individuals during their second or third infection ($P < 0.001$). In parallel, we observed that the titers of anti-PvMSP1₁₉ IgG antibodies in serum samples from individuals during the second or third episode of malaria were significantly higher than the titers of individuals during their first attack of the disease ($P = 0.009$ or $P = 0.013$, respectively). No difference was observed when we compared the antibody titers of individuals during the second and third episode of malaria.

The same type of analysis was performed using recombinant protein ICB2-5 as antigen (Figure 4). We observed that more than 60% of the serum samples had IgM antibodies specific for ICB2-5 during first, second, or third patent *P. vivax* infection. This fact had been previously noted in longitudinal immunoepidemiologic studies carried out in a different endemic area of Brazil.¹⁸ When we compared IgG antibody titers to ICB2-5, we found that individuals in their second or third episode of malaria did not have higher antibodies titers than subjects during their first episode of the disease. In fact, individuals in their third episode of the disease had lower IgG antibody titers ($P < 0.05$).

DISCUSSION

In the present study, we evaluated the persistence of antibody responses to recombinant proteins representing the N- and C-terminal regions of the MSP1 of *P. vivax* in individuals from the city of Belém in the state of Pará in Brazil who had been naturally infected with *P. vivax* malaria. In spite of the fact that we compared antibody titers within two months of infection, specific IgG antibodies to both recombinant proteins decreased significantly in that period in the majority of the individuals. This is the first description of the persistence of the antibody response to PvMSP1 or to any other antigen from *P. vivax*. Previous studies on the longevity of immunity to recombinant proteins of *P. falciparum* MSP1 also reported that during the period of low transmission, the frequency of responders and the level of antibodies were significantly lower.¹⁹ However, this report used only recombinant proteins representing the N-terminal region of PvMSP1 and similar studies involving the C-terminal region have yet to be done.

The rapid decrease in serum antibody levels to PvMSP1

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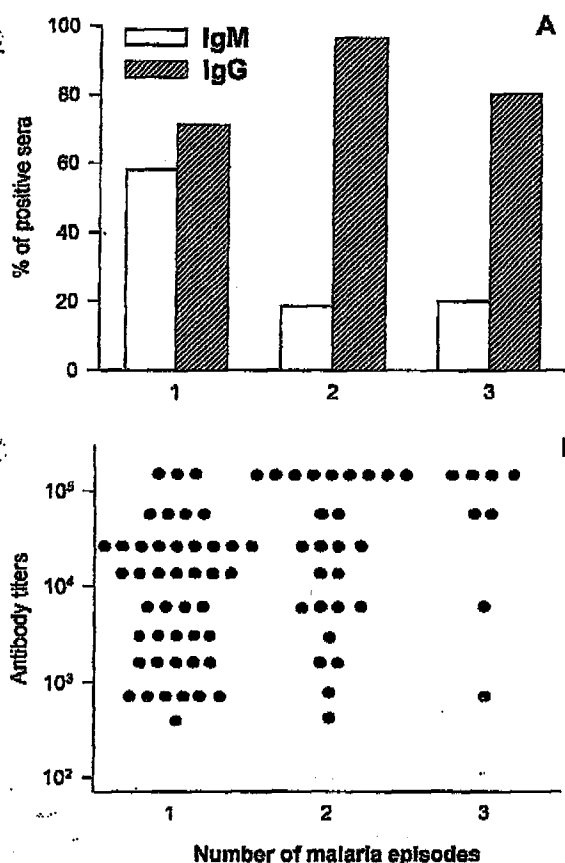


FIGURE 3. Frequency and antibody titers to PvMSP1₁₉ in individuals during the first, second, or third episode of *Plasmodium vivax* malaria. Ninety-nine individuals were grouped according to the number of times they had a *P. vivax* patent infection. The number of individuals of each group was 62, 27 and 10, for one, two, or three episodes of malaria, respectively. The percentage of responders, cut-off values, and antibody titers were estimated exactly as described in Figure 1. A, the frequency of sera from individuals in their first episode of malaria containing specific IgM antibodies was higher than in individuals in their second or third episode of the disease ($P < 0.001$, by chi-square test). B, there was a significant increase in the IgG antibody titers in individuals in their second or third episode of malaria when compared with individuals in their first episode of the disease ($P = 0.009$ or $P = 0.013$, for the second or third episode of the disease, respectively, by Kruskal-Wallis one-way analysis of variance).

in most individuals and perhaps to other malarial antigens contrasts with the slow decrease or maintenance of antibody levels in other diseases. For example, studies of the longevity of the antibody response to filamentous hemagglutinin and pertussis toxin carried out in patients that experienced an outbreak of *Bordetella pertussis* infection demonstrated that during the convalescent phase of the infection, the patients, as well as the hospital medical staff, had high antibody titers to both proteins.²⁰ However, these levels were dramatically reduced after a five-year period in patients that had no further contact with this pathogen, whereas the medical staff who continued to be exposed to this pathogen

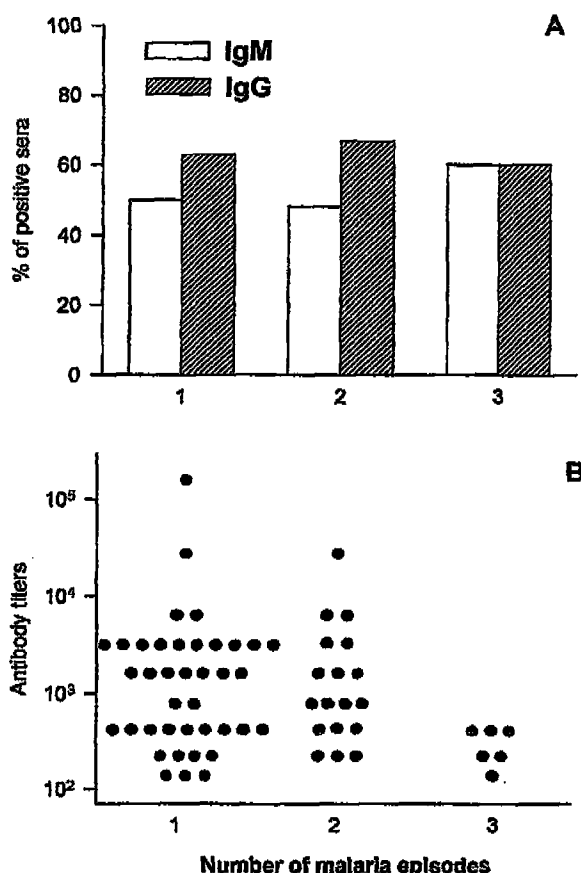


FIGURE 4. Frequency and antibody titers to recombinant protein ICB2-5 in individuals during the first, second, or third episode of *Plasmodium vivax* malaria. Ninety-nine individuals were grouped according to the number of times they had a *P. vivax* patent infection. The number of individuals belonging to each group was the same as described in Figure 3. The percentage of responders, cut-off values and antibody titers were estimated exactly as described in Figure 1. No statistically significant difference was observed among the frequency of positive sera in the different groups of individuals (A). There was no significant increase in the antibody titers in individuals in their first, second, or third episode of malaria (B). Individuals in their third episode of the disease had lower IgG antibody titers ($P < 0.05$).

maintained the antibody levels to both proteins during this same five-year period.²⁰ Moreover, a single infection with smallpox virus generates antibody titers that can be detected for decades.²¹

The reason why antibody production is differentially sustained after infection with distinct pathogens is currently unknown. Several parameters of the host immune response, as well as the nature of the infection, may influence the longevity of the antibody response. They include the persistence of the antigen on follicular dendritic cells, the generation of long-lived plasma cells, or reduced levels of plasma cells apoptosis.²¹ Alternatively, T cells have been described as having a major role in controlling the survival of antigen-specific B cells (plasma cells and memory B cells). Through

8

the interaction of CD40/CD40L, T cells rescue B cells from apoptosis during the initial phase of the immune response.²² Subsequently, they drive the multiplication of B cells in the germinal centers and the isotype switch. Later, however, T cells can be implicated in the elimination of activated B cells by a *Fas* ligand (*FasL*)-mediated destruction.^{23,24} Some of these hypothesis are amenable to be tested in future studies.

To determine why antibody titers decrease after treatment, we initiated the evaluation of the interaction between B and T cells in the generation of antibody immune response to PvMSP1 during natural human infection with *P. vivax*. For that purpose, we determined the frequency of individuals with IgM antibodies specific for these recombinant proteins during the first, second, or third episode of the disease. We also studied whether the antibody titers in individuals at the time of their second or third episode of the disease were higher than in individuals during their first contact with the parasite.

We found that the antibody response to each recombinant protein representing different regions of PvMSP1 was unique. Only a small ($\leq 20\%$) fraction of individuals had IgM antibodies specific for PvMSP1₁₉ during the second or third episode of the disease, whereas the frequency of individuals with IgM to recombinant ICB2-5 was high in all three groups. The observation that individuals were unable to switch the N-terminal-specific antibodies from IgM to IgG had been previously described in longitudinal studies performed in a distinct endemic area.¹⁸ In addition, in the present study, we were unable to observe any evidence for a secondary antibody response to this polypeptide, further suggesting that the interaction between B and T cells during the immune response to the N-terminal region of PvMSP1 is dramatically impaired.

Both an impaired switch from IgM to IgG and the lack of memory cells can be explained by a deficient interaction of CD40/CD40L molecules. This interaction is known to be crucial for the antibody switch in humans because individuals who fail to express CD40L in activated T cells develop X-linked hyper-IgM.²⁵ Similarly, knockout mice that do not express CD40 or CD40L have a profound defect in the production of switched isotypes.^{26,27} The CD40/CD40L interaction is also implicated in the generation of memory B cells.²² Therefore, it is possible that either CD40, CD40L, or both are not properly expressed after activation of B or T lymphocytes by the N-terminal region of PvMSP1.

The discrepancy observed in the antibody response to the N- and C-terminal regions of PvMSP1 may be due to the form of antigenic organization that they are presented to the immune system. Earlier studies have demonstrated that B cell activation is extremely dependent on antigen structure.²⁸ A highly repetitive form of the glycoprotein of vesicular stomatitis virus (VSV-G) expressed on the viral surface is an extremely potent activator of antigen-specific B cells *in vivo*.²⁸ In contrast, poorly organized forms of VSV-G, e.g., a soluble protein, are poor activators of B cells. Because proteolytic cleavage releases the N-terminal of MSP1,^{29,30} it is possible that this region of the molecule is presented to the immune system as a soluble protein (poorly organized antigen). On the other hand, MSP1₁₉ that is maintained on the parasite membrane during the entire process of invasion

may be presented as an organized repetitive epitope leading to a stronger activation of B cells.

The rapid decrease in antibody levels in most individuals to PvMSP1 did not predict a reduction of antibody levels to other malarial antigens. Several donors treated for malaria who had become serologically negative to PvMSP1 had antibodies to other recombinant proteins generated from distinct genes¹ cloned from *P. vivax* (da Cunha MG, unpublished data). These results suggest that antibodies to PvMSP1 alone do not reflect the immune response to all blood-stage antigens of *P. vivax*. This fact is corroborated by findings that there is no correlation between the antibody levels to recombinant proteins ICB2-5 or PvMSP1₁₉ and antibody titers to blood stages as detected by immunofluorescence.³¹ Together, they suggest that immune responses to distinct antigens of *P. vivax* can be differentially regulated. Further studies are underway to dissect them.

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Naturally Occurring Plasmodia-Specific Circulating Immune Complexes in Individuals of Malaria Endemic Areas in India

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Blood samples collected from individuals belonging to malaria endemic areas were assayed for antigen-specific circulating immune complexes in polyethylene glycol precipitates of serum by enzyme immunoassay. Sera were tested from patients with acute *P. vivax* and *P. falciparum* infections, from clinically immune individuals and also from healthy normals. Circulating immune complexes (CICs) containing immunoglobulin G and M isotypes were found to be abundant in individuals with ongoing and past infections and also in clinically immune donors. In patients with acute infection but without any past history of malaria, CICs of IgM type were found to be significantly higher. Demonstration of antigen/antibody specific CICs could be a useful indicator of active, ongoing and recent/past infection, also of the status of immune responses of individuals belonging to various endemic areas.

Keywords: Enzyme immuno assay, Immune complexes, Immunoglobulins, Malaria

INTRODUCTION

Plasmodium vivax and *Plasmodium falciparum* are the two main species responsible for nearly 65% and 35% of malaria in India. Protective immunity to malaria is primarily a premu-

nition, i.e. a resistance to super-infection and general immune responses in malaria infection is evoked through both the effector limbs of the immune system.¹ Humoral as well as cellular immune responses are adequately elicited in malaria infection.^{2,3}

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However, the evasive strategies of *Plasmodium* spp to evade and modulate the immune responses of the infecting host have contributed immensely to their extended survival and propagation in human hosts. In spite of effective elicitation of immune responses, acquisition of sterile immunity is rare, and relapses and recrudescence do exist in malaria. Mechanisms of slow development of immunity in individuals living in endemic areas is also not clearly understood.⁴

Continual exposure of the host to a variety of antigens and subsequent polyclonal activation of B-cells⁵ contribute grossly in the formation of immune complexes (ICs) in the circulation. *In vivo* immune complex formation is a continuous process whenever an antibody meets the antigen, whether specific or non-specific. Several viral, bacterial and parasitic diseases demonstrate immune complex formations; malaria is one of them. Formation of immune complexes in malaria may be one of the outcomes of persistent infection. Low-grade persistent infection and poor antibody response jointly lead to the formation of immune complex and chronicity of the disease. Circulating immune complexes (CICs) have been documented to play an essential role in modulation of the immune response to various antigens.⁶ Their presence in murine, rodent, simian and human malaria have been reported.^{7,8} However, in human malaria very little is known about CICs and antigens and the antibodies involved in their formation.

In the present study, an attempt has been made to determine the plasmodial CICs of IgG and IgM classes directed against *P. vivax* and *P. falciparum* antigens.

MATERIALS AND METHODS

Parasite antigen preparation

***P. falciparum* antigen:** The Indian *P. falciparum* isolate from Delhi, FDL-B was used for antigen preparation, as described earlier.⁹ Parasites were grown and adapted *in vitro* using O + RBCs and AB + serum by the candle jar technique. The culture was harvested when parasitaemia reached almost 10% and a large number of late trophozoites and schizont stages were observed. Parasites were freed by saponin lysis and a soluble extract was obtained by sonication in MSE Soniprep at 14 μ A for 90 sec. The antigen was purified after affinity adsorption with rabbit anti-human erythrocytes antibody, and the protein concentration was determined.

***P. vivax* antigen:** Blood samples were collected from patients who visited the malaria clinic of MRC, Delhi and suffering from *P. vivax* infection after obtaining consent for venipuncture. Nearly 5–10 ml blood was collected aseptically from patients showing 1% or more parasitaemia in heparinised tubes.

Plasma was aspirated and stored frozen; RBC pellet was resuspended in an equal volume of sterile PBS. The suspension was overlayed on 3% dextran sulphate solution, then incubated for 45 min at 37 °C keeping the tube in a slanting position. The supernatant containing WBCs was removed, and the RBC pellet was washed thrice in PBS. The antigen was prepared by saponin lysis, followed by centrifugation, sonication and affinity adsorption.

Collection of blood samples: Blood samples for sera were collected during the transmission season (April to November 1996) from individuals living in various endemic pockets in Delhi, Ghaziabad (PHC-Loni) and belonging to age groups of 15–50 yr. Study subjects were categorised under four groups according to their malaria experience. Blood samples collected from four groups were as: patients suffering from acute *P. vivax* infection and with a history of repeated *P. vivax* and *P. falciparum* infections ($n = 29$); patients with acute *P. falciparum* infection without any history of past infection ($n = 24$); clinically immune adults who earlier suffered repeatedly from malaria but did not show any clinical symptoms at the time of blood collection ($n = 15$); and healthy adults who were residents of endemic zones but without any history of malaria ($n = 20$). A volume of 1–2 ml blood was collected aseptically from each individual after obtaining informed consent. Patients diagnosed for malaria were treated with the recommended doses of chloroquine by the clinician. Sera were separated and stored in aliquotes at -20°C for their use in a number of studies.

Isolation of CICs: Circulating immune complexes were isolated by precipitating at 2.5% (w/v) polyethylene glycol (PEG), mol wt 6000 (SISCO Research Laboratories, India) following the published method.¹⁰ Briefly, 100 μl of serum was diluted five times in PHS-EDTA (0.15 M PBS, pH 7.2 and 0.01 M EDTA, pH 7.6). Equal volumes of freshly prepared 5% PEG in PBS were added to the diluted serum, then incubated overnight at 4°C . The precipitates were obtained by centrifuging at $1000 \times g$ for 30 min at 20°C , then washed once with

2.5% PEG. Finally, precipitates were reconstituted to the original volume by dissolving thoroughly in PBS and kept at 37°C for 2 h. The dissolved precipitates were stored at -20°C till use.

Detection of malarial antigen specific CICs:

The CICs in PEG precipitates were detected by simple indirect ELISA. Polystyrene round-bottom 96 well ELISA plates were coated separately with 50 μl per well *P. vivax* and *P. falciparum* crude antigens diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 at a concentration of 10 $\mu\text{g}/\text{ml}$, then they were incubated overnight at 4°C . After removing excess antigens, the plates were washed twice with PBS-Tween-20 (PBS-T) and blocked with 1% BSA for 2 h at 37°C . PEG precipitates diluted in PBS at a dilution of 1:100 (50 μl) were added in quadruplicate wells in both *P. vivax* and *P. falciparum* antigen coated plates and incubated overnight at 4°C . After washing five times with PBS-T, anti-human IgG and IgM conjugated with HRPO were added in respective wells (50 μl) in duplicate and incubated for 1 h 30 min at 37°C . Enzyme-specific substrate, orthophenylene di-amine/ H_2O_2 (50 μl) was added in wells after washing five times with PBS-T and kept for reaction in the dark for 30 min at room temperature. Enzyme-substrate reaction was terminated by adding 50 μl of 8 N sulphuric acid and the plates were read at 492 nm in an ELISA reader (Titertek Multiskan Plus, Flow Laboratories, UK).

Statistical analysis: Mean ELISA O.D. values of each group were compared using student's *t-test* and probability values for the

532
532

CICs in each group were determined.

RESULTS

Sera tested in this study were collected from a group of individuals suffering from both *P. vivax* and *P. falciparum* malaria. Some of them had multiple attacks, while some of them had no history of malaria infection. The IgG and IgM type CIC profiles of these samples detected against *P. vivax* and *P. falciparum* antigens are shown in Fig. 1. In healthy normals (Group A), *P. vivax* and *P. falciparum* specific IgG and IgM type immune complexes were found to be much

lower. A few samples demonstrated slightly higher values in both cases. In *P. vivax* patients (Group B), CICs of both the Ig isotypes were found to be elevated when tested against two antigens, indicating their present and recent past infections. The patients with acute infection of *P. falciparum* (Group C) demonstrated significantly higher IgM type CICs with *P. falciparum* antigen since these patients were suffering from a fresh bout of *P. falciparum* infection. In the clinically immune donors (Group D), CICs of IgM type were substantially high as observed by the reactivity of PEG ppt with both *P. vivax* and *P. falciparum* antigens. These may indi-

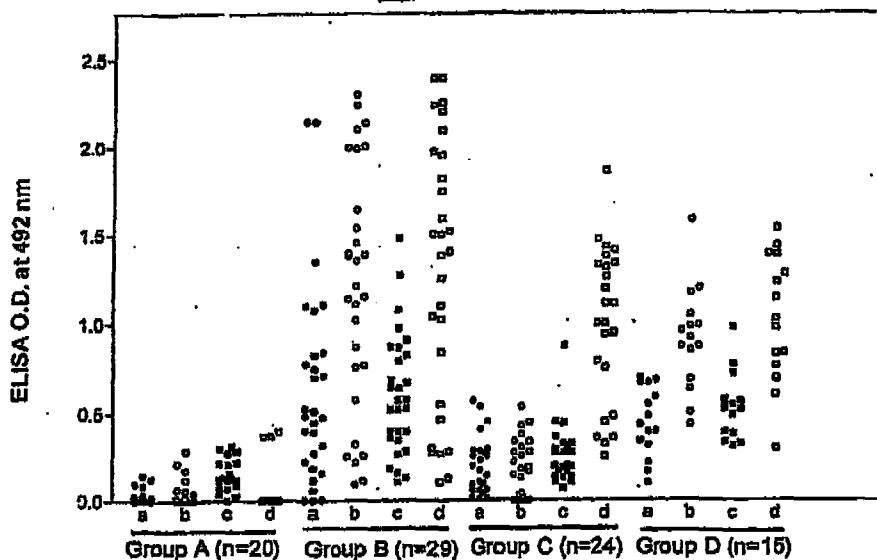


Fig. 1: Scatter diagram of plasmodial-specific CICs of IgG and IgM classes in sera from individuals.

Groups are normal healthy control (A), acute *P. vivax* and *P. falciparum* patients (B and C), clinically immune individuals (D). The graph is plotted with ELISA O.D. values at 492 nm.

Profile of CICs: a = IgG type with *Pv* Ag; b = IgM type with *Pv* Ag; c = IgG type with *Pf* Ag; d = IgM type with *Pf* Ag.

14

Table 1. Plasmodial (*P. vivax* and *P. falciparum*) CICs (IgG and IgM types) in PEG precipitates of sera from healthy normals, patients and immune individuals

Group	Sample size	ELISA O.D. values with <i>Pv</i> Ag		(Mean $\pm$ S.D.) with <i>Pf</i> Ag	
		IgG	IgM	IgG	IgM
A (Healthy)	20	0.03 $\pm$ 0.04	0.05 $\pm$ 0.08	0.15 $\pm$ 0.09	0.06 $\pm$ 0.14
B (<i>Pv</i> case)	29	0.62 $\pm$ 0.55	1.25 $\pm$ 0.80	0.60 $\pm$ 0.34	1.29 $\pm$ 0.75
C (<i>Pf</i> case)	24	0.20 $\pm$ 0.12	0.23 $\pm$ 0.16	0.22 $\pm$ 0.09	1.04 $\pm$ 0.41
D (Immune)	15	0.45 $\pm$ 0.19	0.91 $\pm$ 0.28	0.54 $\pm$ 0.18	1.03 $\pm$ 0.34
Statistical analysis					
A vs B		NS	NS	p=0.05	p=0.05
A vs C		p<0.05	p<0.05	p<0.001	p<0.001
A vs D		p<0.01	p<0.001	p<0.001	p<0.001

NS: Not significant.

cate that clinically immune individuals might be harbouring submicroscopic/subclinical infection in the system, which, in turn, provides immunity to the disease. These results with their statistical significance are shown in Table 1.

DISCUSSION

Observations of elevated levels of plasmodial CICs of IgG and IgM types in endemic areas are understandable as intense exposure to malaria may elicit humoral immune response in individuals residing in endemic areas.^{11, 12} Immune complexes are formed during the immune response to an introduced or exposed antigen by binding to the newly formed specific antibodies to that antigen.¹³

The endemic areas where the study subjects belong offered ideal malariogenic conditions. In

these areas malaria is seasonal: early, prolonged monsoons are responsible for intensive transmission of both *P. vivax* and *P. falciparum*. Coexistence of both *P. vivax* and *P. falciparum* CICs in individuals may indicate the high prevalence and infestation rate of these species in the community. Malariometric surveys conducted in northern India have shown the presence of both *P. vivax* and *P. falciparum* in many patients.¹⁴ There are reports in which cross-reactivities among different strains of *Plasmodium* have been demonstrated^{15,16}. These studies suggest that certain antigens — or at least some of their epitopes — might be common to different species of plasmodia. High levels of IgM-class CICs directed to *P. falciparum* antigens in patients with clinically acute *P. falciparum* infection may indicate their first encounter to this infection, as IgM is the predominant immunoglobulin produced during the primary immune res-

ponse¹⁷. Immune individuals have shown high IgM-type CICs which may be due to subclinical infection consisting of a new clonal subpopulation of parasites with a very low level of parasitaemia. This group of individuals was also having CICs of IgG type, but their level was found to be lower than the IgM-type complexes. The continuous boosting of the immune system with low-grade parasitaemia might be helping in acquisition of immunity. High levels of IgM in areas of fresh malaria outbreak have also been reported.¹⁸ The polyclonal B-cell activation by plasmodial antigens produces arrays of antibodies which may not be protective to the host. The high load of CICs detected in sera may be both protective and non-protective antibody-bound complexes. It is important to identify antigen-antibody complexes relevant to the development of host protective immunity in people living in malaria endemic areas of India. In various species including human, ICs are formed during the immune response. A small proportion of the ICs is trapped in B-cell follicles on the cellular processes of follicular dendritic cells and can be retained there for a long period of time¹⁹ via binding to Fc receptor²⁰ and complement C3 receptor.²¹ Antigen preserved in this way is believed to play a crucial role in the generation of memory B-cell and the maintenance of long-term humoral immune responses. *In vitro* performed ICs have been shown to be much more efficient in inducing humoral immune responses *in vivo* than the native antigen.²² This specific potential of ICs may suggest their use as vaccine antigens. Presence of CICs in clinically immune population living in endemic areas may explain the low infection rate in these individuals.

It may be concluded that determination of CICs in endemic areas could provide valuable data for evaluation of active transmission, current infection rate as well as the immune response profile of the host.

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13

536

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Evaluation of Indirect Immunofluorescent Antibody Test for Detection of IgM Specific Antibodies in Malaria

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ABSTRACT

Indirect immunofluorescent antibody test using *Plasmodium falciparum* antigen from *in vitro* culture was evaluated for detecting IgM antibodies in order to determine the feasibility of its application in serodiagnosis of malaria. Test was compared with the already adapted IgG-IIF test using the same antigen. It was found that none of the healthy controls and slide negative fever cases had malaria IgM antibodies whereas 8 per cent of healthy controls and 49.01 per cent of the slide negative fever cases had malaria IgG antibodies. The sensitivity of IgM-IIF test was 94.68 per cent and that of IgG-IIF test was 96.81 per cent. IgM antibodies could be detected very early even on the first day of fever and titre rose gradually with increasing number of days of illness before institution of treatment. The IgM antibodies, being short lasting are able to reflect recent infection. The test although highly sensitive and specific is laborious and expensive. Therefore, it may be used as a serodiagnostic test in advanced laboratories only for confirmation of selected slide negative cases.

INTRODUCTION

Malaria infection is best diagnosed by careful microscopic examination of a stained thick blood smear. This is simple and does not require any sophisticated equipment. One of the disadvantages of this method is that it is time consuming and with large number of samples the accuracy cannot be ensured. In addition, due to easy availability of antimalarials at present, parasites may not be detectable in the peripheral smear and diagnosis cannot be ascertained in some cases. Indirect immunofluorescent test (IIF) is a well established test for detecting malaria antibodies and has been used considerably for epidemiological purposes¹⁻². By this test either IgG antibodies or total immunoglobulin response is conventionally determined. These antibodies are long lasting and cannot be of help in the serodiagnosis of malaria in an endemic country like

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538

Table 1—Malaria-IgM antibody titres in malaria and control sera by IIF test

Sera tested	Total	< 16	16	32	64	256	1024	4096	Total >16	% >16
<i>Malaria Sera</i>										
Total	94	5	16	24	34	10	3	2	89	94.68
<i>P. vivax</i>	70	5	16	23	22	4	0	0	65	92.86
<i>P. falciparum</i>	24	—	—	1	12	6	3	2	24	100
Healthy control	50	50	—	—	—	—	—	—	—	0
Slide negative fever cases	51	51	—	—	—	—	—	—	—	0

Table 2—Malaria-IgG antibody titres in malaria and control sera by IIF test

Sera tested	Total	< 32	32	64	256	1024	4096	Total > 32	% > 32
<i>Malaria Sera</i>									
Total	94	3	3	18	24	14	32	91	96.81
<i>P. vivax</i>	70	3	3	18	24	7	15	67	95.71
<i>P. falciparum</i>	24	—	—	—	—	7	17	24	100
Healthy control	50	46	4	—	—	—	—	4	8
Slide negative fever cases	51	26	18	8	7	—	—	25	49.02

The mean IgM and IgG malaria antibody titre (GMT) with S.D. during 1st day, 2-5 days, 6-10 days and 11-30 days of illness is presented in Table 3. The student "t" test was applied to determine the significance of rise in GMT between these specified periods. The GMT of IgM was very low on the 1st day of fever which rose significantly after 2-5 days ($t = 2.41, p < 0.05$). The rise was highly significant after 6-10 days ($t = 3.79, p = < 0.001$) and after 11-30 days ($t = 4.07, p = < 0.001$). The GMT of IgG was quite high even in those giving history of 1 day fever (340.56). Although there was an apparent rise in GMT during the illness up to 30 days, the rise was significant only after 11-30 days after infection ($t = 3.14, p = < 0.01$)

DISCUSSION

The results of the present study confirm earlier reports that both malaria specific IgM and IgG responses occur in malaria.^{3, 4} IgM antibodies were not detected in healthy controls and slide negative fever cases. However, in these groups, malaria specific IgG antibodies were detected. This observation confirms earlier findings⁵ that duration of IgM response in malaria is transient as compared to the IgG antibodies which persist for a long time specially after repeated

70

IIFAT—IgM antibodies in malaria

85

Table 3—Geometric mean titre (GMT) of IgM IgG in malaria cases by IIF test

Type of Malaria Antibody	GMT with 95% Confidence Interval of malaria antibody in patients with number of days of illness				Total
	1 day	2-5 days	6-10 days	11-30 days	
IgM	25.06 (17.88, 35.28)	43.61 (35.02, 54.57)	103.97 (50.58, 213.78)	219.45 (64.89, 744.43)	50.67 (39.69, 64.45)
IgG	840.66 (149.09, 778.05)	409.74 (259.57, 648.07)	1176.27 (413.0, 3350.13)	2580.32 (1408.55, 4705.07)	631.66 (367.09, 765.36)

infections. Therefore, the IgM-IIF test appears to be very specific as an immunodiagnostic test.

The sensitivity of the IgG IIF test was 97 per cent in this study and was higher than that reported in our earlier studies on IIF test.¹² This figure is however comparable to reports by previous workers using Aotus *P. falciparum* antigen.¹³ This improvement in sensitivity could be due to the use of a synchronous *P. falciparum* culture containing mainly schizonts as antigen in this study. Out of the same malaria cases 94.68 per cent were having malaria specific IgM antibodies showing high sensitivity of this test. In those with *P. vivax* parasitaemia the sensitivity was 93 per cent and in those with *P. falciparum* parasitaemia 100 per cent were seropositive. The difference could be because of use of *P. falciparum* as antigen. The very high sensitivity of the test again makes it very suitable as an immunodiagnostic test.

Both the IgM and IgG levels rose with increasing number of days of infection before institution of treatment showing that longer duration of antigenic exposure increased the level of antibodies.

In the present study, IgM antibodies could be detected very early in infection. Cases with history of fever even for one day, showed very low levels of IgM antibodies. High levels of IgG antibodies were also found on the first day of infection indicating that these antibodies persisted from earlier infections due to their long lasting nature.

Thus, IgM-IIF test gives information regarding recent infection and may be utilized as a tool for surveillance in the near eradication phase. The limitations of the test are that it is laborious and can be performed only for selected cases in a few sophisticated laboratories having facilities of fluorescent microscopy.

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325

Structure and expression of the gene for Pv200, a major blood-stage surface antigen of *Plasmodium vivax*

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Molecular cloning and structure analysis of the gene encoding the Pv200 protein of the Sal-I strain of *Plasmodium vivax* revealed an overall identity of 34-37% when the deduced amino acid sequence was compared with the sequences of various major merozoite surface antigens of *Plasmodium falciparum*, *Plasmodium yoelii* and *Plasmodium chabaudi*. When the Sal-I Pv200 sequence was compared with the corresponding sequence from the Belém strain of *P. vivax*, it was found that the two merozoite surface antigens were relatively well conserved with an overall amino acid sequence identity of 81%. A region of 23 repeated glutamine residues, found in the sequence of the Belém isolate was not found, however, in the Sal-I sequence. Amino- and carboxy-terminal domains of the Pv200 protein were expressed in the yeast *Saccharomyces cerevisiae*. Each recombinant protein was shown to react with antibodies in sera from splenectomized Bolivian *Saimiri* monkeys that had been infected previously with *P. vivax*, and in human sera from individuals with a history of exposure to vivax malaria. The availability of recombinant DNA-derived Pv200 proteins will now allow a full assessment of their utility in the diagnosis and immunoprophylaxis of the benign tertian malaria associated with *P. vivax* infection.

Key words: *Plasmodium vivax*; Blood-stage antigen; Pv200; Malaria vaccine

Introduction

Large scale in vitro parasite culture cannot provide sufficient quantities of either organisms or antigens to produce a malaria vaccine. Accordingly, peptide synthesis and recombinant DNA methodologies are being evaluated

extensively for the production of potential protective immunogens against the protozoan parasites that cause malaria. Because of the severity of the disease caused by *Plasmodium falciparum*, this species has represented the major target for such studies. Of the numerous potential subunit vaccine candidates from this organism, an antigen of special interest has been the major merozoite surface antigen Pf195. This antigen has been shown to reside on the surface of the schizont and, in a processed form, on the surface of the merozoite [1]. Vaccination studies with Pf195, isolated from cultured parasites, have led to high levels of protection against *P. falciparum* challenge in monkey model systems [2]. More recently, recombinant DNA-derived Pf195 antigens have been produced and studied as candidate vaccines against falciparum malaria [3-5].

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Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession number M75674.

Abbreviations: ADH2, alcohol dehydrogenase-2; CS, circumsporozoite; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TMD, transmembrane domain.

Although less virulent than *P. falciparum*, *Plasmodium vivax* is the causative agent of benign tertian fever, a form of malaria characterized by frequent and protracted relapses. Vaccination studies against this species have been less extensive than with *P. falciparum* and have been focused almost exclusively on the sporozoite lifecycle stage of the organism [6-9]. Recently, however, a polymorphic 200-kDa component of the *P. vivax* schizont surface was defined by monoclonal antibodies, and a partial genomic clone that encoded a portion of the antigen was isolated, structurally defined and expressed in bacteria [10]. Homology of this DNA fragment, and the more recently isolated full length gene sequence [11], with the Pf195 gene, together with immunolocalization studies using antisera to the expressed protein have suggested strongly that the Pv200 protein is functionally analogous to Pf195 [10]. Here we report the molecular cloning and structure analysis of the gene for Pv200 from the Sal-1 strain [8] of *P. vivax*. We also demonstrate that amino- and carboxy-terminal domains of the protein produced in yeast can be used to detect antibodies both in monkeys and in humans previously infected with *P. vivax*.

Materials and Methods

Construction of *P. vivax* genomic DNA libraries. Genomic libraries were prepared as follows. 500 ng of *P. vivax* genomic DNA, from the Sal-1 strain, was digested with *Eco*RI and ligated into *Eco*RI digested λ ZAPII (Stratagene), packaged and introduced into *Escherichia coli* strain PLK-17. A similar *Hind*III digest, partially filled with dCTP and dTTP, was ligated into *Xba*I digested λ ZAPII that had been similarly filled with dATP and dGTP. The ligated DNA was packaged and transformed as above. Libraries of 5×10^7 and 4.1×10^7 independent clones were obtained, respectively.

Screening of *P. vivax* DNA. Two overlapping oligomers (45-mers), based on the sequence of

del Portillo et al. [10], were labeled by the oligomer primed extension method [12], hybridized in 40% formamide-containing buffer [13] at 37°C to a Southern blot of *Eco*RI-digested *P. vivax* DNA, and washed at 65°C in $2 \times \text{SSC}/0.1\%$ SDS. A 9.5-kb *Eco*RI fragment hybridized to this probe. Two overlapping oligomers (42- and 43-mers) based on the 5'-end of the *Eco*RI clone were used to probe a Southern blot of *Hind*III-digested *P. vivax* DNA in a similar fashion and hybridized to a 7.0-kb fragment. Similar hybridizations were carried out on library filters and plaque purified tertiary positives were excised using *E. coli* strain XLI-Blue (Stratagene) and plasmid DNA was retransformed into *E. coli* strain D1210 for further plasmid manipulations.

Subcloning and DNA sequencing. Plasmid DNA was isolated by the alkaline lysis method [13]. Overlapping restriction fragments were subcloned into M13 vectors and both strands were sequenced by the chain-termination method [14] using M13 primers as well as specific internal primers. DNA manipulations were essentially as described [13].

Expression of amino- and carboxy-terminal domains of Pv200 in *Saccharomyces cerevisiae*. The polymerase chain reaction (PCR) [15] was used to amplify DNA fragments from cloned Pv200 gene sequences. Appropriate restriction sites, and in-frame initiation and termination codons were incorporated into the PCR primers. Thus, for Pv200A, primers 5'-(dA-TGTCCTCATGGAAACAGAAAGTTATAA-GCAG)-3' and 5'-(dCGCCCTCAACAAATCATAGTG)-3' were used to amplify an *Nco*I/*Eco*RI fragment from the *Hind*III clone 4B-3-9. A pBluescript polylinker primer, 5'-(dGTGGATCCCCCGGGCTGCAGG)-3' and the 3'-primer 5'-(dTTCGAAGGTCGAC-TATGGATTTTGCAAATCACCAATGT)-3' were used to amplify the contiguous *Eco*RI/*Sal*I fragment from the *Eco*RI clone 6.1-2. The PCR products were digested with the appropriate restriction enzymes and ligated into *Nco*I/*Sal*I digested pBS100 [7]. A *Bam*HI/*Sal*I fragment that contained the ADH2/GAPDH

hybrid promoter [16] fused to the cloned PCR product was excised and cloned into pBS24 [17] for yeast expression. The Pv200B gene was amplified as an *NcoI/SalI* fragment from clone 6.1-2 using the primers 5'-(dCGGGAGCC-ATGGCAGAATCTGAGGCGCCTGAG)-3' and 5'-(dTTC CAAGGTCGACTAGCTAC-AGAAA ACTCCCTCAAAGAG)-3'. Subcloning into pBS100 and pBS24 were as above. pBS24 plasmids containing the Pv200A and Pv200B genes were transformed into *Saccharomyces cerevisiae* strain JSC302, a derivative of AB116 [18] that overexpresses the *ADRI* gene [16]. Transformants were propagated and induced as described previously [7]. Yeast cell lysates were analyzed on 12.5% SDS-PAGE gels with Coomassie Blue staining, and by immunoblotting.

Purification of recombinant proteins. Yeast cells that were expressing Pv200A were harvested at 60 h and lysed with glass beads in 1 M NaCl/50 mM Tris/1 mM EDTA/0.1% Triton X-100 at pH 7.5 [7]. After centrifugation, Pv200A was extracted from the insoluble pellet with 8 M urea/100 mM Tris (pH 11.5). The solution was adjusted to pH 8.3 and passed over a Mono Q (Pharmacia) anion exchange column in 6 M urea/50 mM Tris-HCl/1 mM EDTA. Pv200A was found to be >95% pure by densitometric scanning of a Coomassie Blue-stained SDS-PAGE gel. Yeast cells expressing Pv200B were harvested and lysed in a similar manner. The 8 M urea extract was then adjusted to pH 9 and run over a Fast Flow Q (Pharmacia) ion exchange column. The immunoblot-positive peak eluting at 0.15–0.20 M NaCl in a 0–1 M gradient was collected, concentrated and separated by gel filtration on Superose 12 resin (Pharmacia) in 4 M urea/10 mM Tris/1 mM EDTA/10 mM β -mercaptoethanol at pH 8.

Serology. Immunoblots and ELISA assays on monkey and human sera were performed essentially as described [13]. Monkey sera were obtained from *Saimiri boliviensis* squirrel monkeys that had been used previously as controls to assess the efficacy of a recombinant

P. vivax circumsporozoite vaccine [8,9]. Anti-*Cynomolgus* conjugates were used to assay antibodies in monkey samples. Sera were taken 4 weeks after challenge with 10 000 *P. vivax* Sal-I sporozoites. Human sera were collected from a population in Brazil, outside the endemic zone, that had encountered a single 50-day exposure to *P. vivax* [19]. Serum samples were taken 8 months after the outbreak, which was completely controlled by chemotherapy and insecticides. Enhancement of sensitivity in the human sera ELISAs was achieved using the 3, 3', 5, 5'-tetramethylbenzidine (TMB) as substrate and reagents as recommended by the manufacturer (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD).

Results and Discussion

Molecular cloning of the Pv200 gene. Overlapping λ ZAPII phage clones were isolated that contained the large open reading frame encoding Pv200 (Fig. 1). Most of the Pv200 protein sequence is encoded by the *EcoRI* clone (6.1-2). The amino-terminal region is contained within the ca. 7-kb *HindIII* clone (4B-3-9). The full DNA sequence of a 5.83-kb composite *NdeI* fragment that includes the entire Pv200 coding sequence is shown (Fig. 2). Typical promoter elements [20] are found within the 5'-region of the sequence, and a consensus motif for efficient initiation of translation [21] is apparent around the proposed initiation codon.

Structure of Pv200. As deduced from the composite sequence, Pv200 is derived from a 1751-amino acid precursor protein that contains a typical amino-terminal secretory signal sequence [22], and a transmembrane domain (TMD) (Fig. 1). A predicted signal peptidase cleavage site [22] occurs after Cys19. Also, within the hydrophobic TMD (Fig. 2), is a stretch of 4 serine residues that is reminiscent of the signal for attachment of glycosylphosphatidyl inositol anchor sequences to proteins of various protozoans, including Pf195 [23].

25

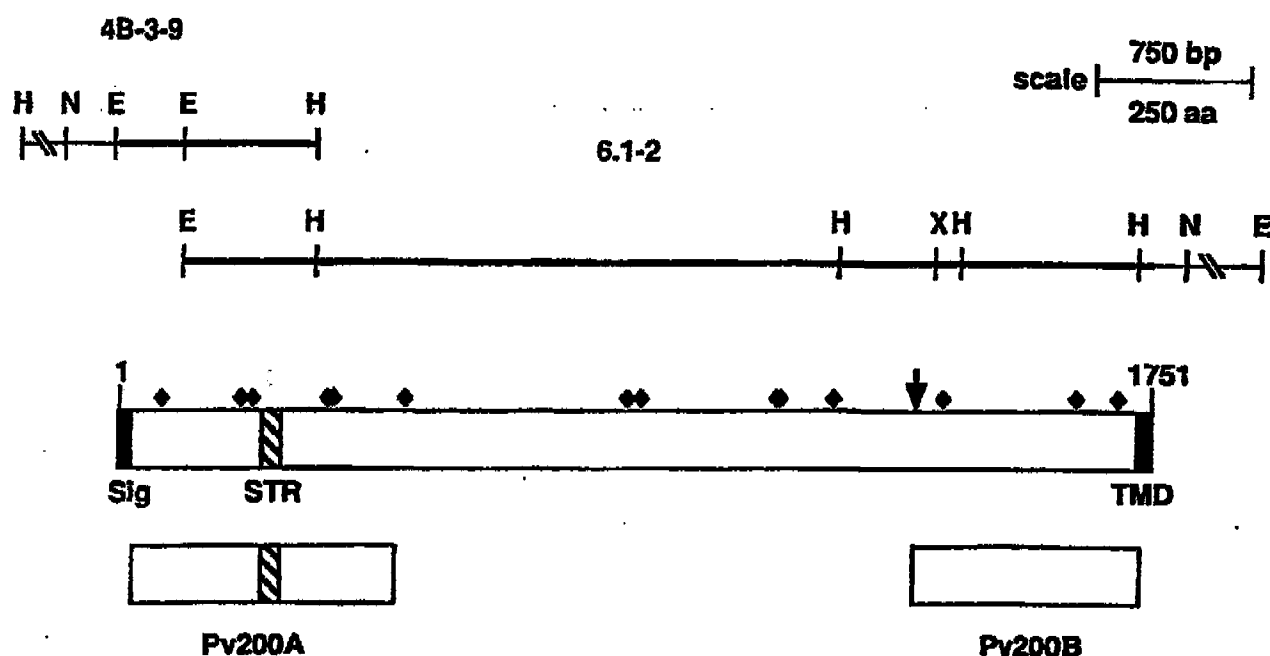


Fig. 1. Restriction maps of the overlapping genomic DNA clones that encode Pv200, shown above a schematic representation of the encoded protein sequence. The flanking *NdeI* sites (Fig. 2, legend) are shown (N). Other sites used for mapping and DNA sequencing: *HindIII* (H), *EcoRI* (E), and *XbaI* (X). Potential asparagine-linked glycosylation sites are shown (◆), as is the serine/threonine-rich region (STR). The signal sequence (Sig) and putative transmembrane domain (TMD) are shown in black, and a potential proteolytic cleavage site (see text) is arrowed. Also shown schematically, are the regions Pv200A and Pv200B that were selected for expression in yeast.

Overall amino acid sequence identity between our Pv200 and Pf195 from various sources [24] is in the 36–37% range, although a region of more extensive identity (45%) exists in the amino-terminal region [10, and present study]. Similarly, amino acid sequence identities between Pv200 and the major merozoite surface antigens of *P. chabaudi chabaudi* and *P. yoelii yoelii* were 34.6% and 34.9%, respectively [25,26]. Also present is a potential proteolytic cleavage site identical to that defined previously for the generation of the carboxy-terminal p42 protein of the Pf195 precursor [27] (Figs. 1 and 2, arrowed). Also in common with Pf195, Pv200 does not contain multiple repetitive elements that are characteristic of many other proteins of malaria parasites. Six serine/threonine-rich motifs of 5 amino acids are noted, however (Figs. 1, 2). Also, the striking 23-glutamine residue repeat of the Belém strain Pv200 protein was not present in the sequence encoded by our cloned gene, but rather, was replaced by a 35-amino acid residue stretch that contained only 6

glutamine residues. This, together with several other smaller insertions and deletions accounts for the larger size of the Sal-1 Pv200 precursor over that of the 1726 amino acid Pv200 precursor from the Belém strain. Despite this size difference, the two Pv200 precursors are relatively well conserved, with an overall amino acid sequence identity of 81%. As with the major merozoite surface antigens from all *Plasmodium* species that have been studied thus far, this intra-species homology could be divided into areas of the protein of relatively

Fig. 2. Composite DNA sequence of a 5.83-kb *NdeI* fragment containing the Pv200 coding sequence. The 1751-amino acid large open reading frame is shown with proposed amino-terminal signal and carboxy-terminal membrane-spanning sequences underlined. Also underlined, are 13 asparagine residues that are potential sites for glycosylation. A 30-amino acid serine/threonine-rich region between amino acids 241 and 270 that contains 6 copies of the 5 amino acid repeat motif G.S.(S/T).(N/S/G).(S/T) is also noted (double arrow). A potential proteolytic cleavage site (see text) after amino acid Glu1356 is arrowed.

