Naturally Acquired Antibody Responses to the C-Terminal Region of Merozoite Surface Protein 1 of Plasmodium vivax in Korea

JAE-WON PARK,1 SEUNG-HWAN MOON,1 JOON-SUP YEOM,2‡ KOOK-JIN LIM,3 MI-JIN SOHN,3 WOO-CHUL JUNG,4‡ YOUNG-JUNG CHO,4 KI-WON JEON,4 WOONG JU,5§ CHANG-SEOK KL,4 MYOUNG-DON OH,6,7 AND KANGWON CHOE6,7

Korean Armed Forces Central Medical Research Institute, Yusong-gu, Daejeon,1 5th Infantry Division of the ROK Army, Jeongok-eup, Yecheon-gun, Kyonggi-do,2 LG Biotech Research Institute II, LG Chemical Ltd. Research Park, Yusong-gu, Daejon,3 Korean Armed Forces Medical Command, Boondang-gu, Seongnam-si, Kyonggi-do,4 Yeoncheon Health & Medical Center, Jeongok-eup, Yeoncheon-gun, Kyonggi-do,5 and Department of Internal Medicine, Seoul National University College of Medicine,6 and Clinical Research Institute, Seoul National University Hospital,7 Chongno-gu, Seoul 110-744, Republic of Korea

Received 4 May 2000/Returned for modification 15 August 2000/Accepted 20 September 2000

We expressed a protein in Saccharomyces cerevisiae in order to evaluate the humoral immune responses to the C-terminal region of the merozoite surface protein 1 of Plasmodium vivax. This protein (Pv200)18 had a molecular mass of 18 kDa and was reactive with the sera of individuals with patent vivax malaria on immunoblotting analysis. The levels of immunoglobulin M (IgM) and IgG antibodies against Pv20018 were measured in 421 patients with vivax malaria (patient group), 528 healthy individuals from areas of nonendemicity (control group 1), and 470 healthy individuals from areas of endemicity (control group 2), using the indirect enzyme-linked immunosorbent assay (ELISA) method. To study the longevity of the antibodies, 20 subjects from the patient group were also tested for the antibody once a month for 1 year. When the cutoff values for seropositivity were determined as the mean + 3 × standard deviation of the antibody levels in control group 1, both IgG and IgM antibody levels were negative in 98.5% (465 of 472) of control group 2. The IgG and IgM antibodies were positive in 88.1% (371 of 421) and 94.5% (398 of 421) of the patient group, respectively. The IgM antibody became negative 2 to 4 months after the onset of symptoms, whereas the IgG antibody usually remained positive for more than 5 months. In conclusion, indirect ELISA using Pv20018 expressed in S. cerevisiae may be a useful diagnostic method for vivax malaria.

Malaria is the most prevalent parasitic disease in the world, and Plasmodium vivax is the second most prevalent species causing malaria, with a yearly estimate of 35 million cases worldwide (11). P. vivax exhibits two distinct types of incubation-relapse patterns, that apparently depend upon its geographical origin. The Chesson strain of New Guinea is a good example of the tropical type of pattern, which is characterized by an early attack, a short latent period, and then a relapse. In contrast, the St. Elizabeth strain of the temperate type exhibits an early primary attack, followed by a long latency of 6 to 11 months. It is thereafter succeeded by a series of relapses occurring at short intervals.

Vivax malaria was an endemic disease in the Republic of Korea (ROK) until the 1970s. However, no indigenous malaria had been reported in the ROK since 1984, and the ROK was considered to be free from malaria at that time. It was not until 1993 that the first reemerging vivax malaria developed near the demilitarized zone (DMZ) in a young soldier who apparently had no history of traveling abroad. Since then, the number of malaria cases has increased exponentially year after year in the northwestern part (the northern part of the Kyonggi Province) of the ROK, reaching more than 1,700 cases in 1997 and approximately 4,000 cases in 1998 (4, 7, 9, 24). One of the characteristics of Korean vivax malaria is a prolonged incubation period, which lasts up to 1 year, in a large proportion of patients (26).

Among the proteins of the erythrocytic stages of Plasmodium, merozoite surface protein 1 (MSPI) has been the most intensively studied as a potential target for protective immunity. This protein is synthesized as a precursor with a high molecular mass (180 to 230 kDa) during the stage of schizogony, and it is later processed into several of the major merozoite surface proteins (16). During the invasion process, proteolytic cleavage releases most of the molecule from the merozoite surface, and only a 19-kDa fragment of the C-terminal region is carried into the invaded erythrocytes (1, 2). The biological importance of MSPI for parasite survival remains to be elucidated. However, it has been well established that antibodies which recognize its C-terminal region inhibit merozoite invasion in vitro (5, 6, 25) and confer passive immunity to naive mice (3). The potential of this molecule for vaccine
development has motivated researchers to study the generation of recombinant proteins containing portions of MSP1. Several recombinant proteins based on the MSP1 sequence of different Plasmodium species have been used to immunize rodents and monkeys. Recent studies have demonstrated that such recombinant proteins can elicit a significant protective immune response (22).

There have been relatively few studies done on the immune response to \textit{P. vivax} infection. The N-terminal region of the MSP1 of \textit{P. vivax} (PvMSP1) has been expressed in \textit{Escherichia coli} (8, 21, 23) and in \textit{Saccharomyces cerevisiae} cells (13). In a study performed in Brazil, it was reported that the N-terminal region of PvMSP1 was immunogenic. However, 40% of the individuals with patent infection did not have detectable levels of immunoglobulin \textit{G} (IgG) to the recombinant proteins representing the N-terminal region of PvMSP1, even after multiple malaria attacks. The N- and C-terminal regions of PvMSP1 were also expressed as glutathione S-transferase fusion proteins, respectively, and the recombinant proteins were tested in Brazilian patients with vivax malaria (30). The results of this study showed that 51.4% of the patients were seropositive for the recombinant proteins representing the N-terminal regions of the PvMSP1, and 64.1% of the patients were positive for the proteins representing the C-terminal regions of PvMSP1. However, immune responses against the PvMSP1 from \textit{P. vivax} of the temperate type have not been studied in detail as of yet.

We expressed the C-terminal region of PvMSP1 in \textit{S. cerevisiae} and measured the IgM levels, as well as the IgG levels against the C-terminal region of PvMSP1, in order to study the humoral immune response to PvMSP1. We also determined the longevity of the immune response to PvMSP1.

\section*{MATERIALS AND METHODS}

\textbf{Subjects.} In order to study the sensitivity and specificity of the antibody test, healthy individuals from areas of nonendemicity (control group 1), healthy individuals from areas of endemicity (control group 2), and vivax malaria patients (patient group) were enrolled in this study. Individuals in control group 1 were recruited from a group of healthy soldiers serving at Daejeon City or Chong-cheon Province, where malaria does not occur. Control group 1 consisted of 528 subjects. Blood samples from these soldiers were collected in late July when the incidence of malaria reaches its peak level in the ROK. Individuals in control group 2 were recruited from a group of soldiers serving near the DMZ, where malaria is endemic. Those who had a history of malaria were excluded. Control group 2 consisted of 472 subjects, and blood samples were also collected in July. All of the subjects in the controls groups were male and between the ages of 20 and 25.

The patient group included 421 soldiers who were admitted to military hospitals because of patent malaria between May 1998 and October 1999. All of these patients were male and between 20 and 25 years of age. All were admitted from 1 to 7 days after the onset of symptoms. Two-thirds were admitted to the hospitals because of patent malaria between May 1998 and October 1999. All of the soldiers in control group 1. Among 472 whole blood samples from control group 2 collected at the area of endemicity, \textit{P. vivax} parasites were detected in two soldiers by nested PCR before the treatment, but were not detected in any of the soldiers in control group 1. Among 472 whole blood samples from control group 2 collected at the area of endemicity, \textit{P. vivax} parasites were detected in two soldiers by nested PCR. These soldiers had no clinical signs or symptoms of malaria at the time of our sampling.

\section*{RESULTS}

\textbf{Detection of \textit{P. vivax} parasites by nested PCR.} \textit{P. vivax} parasites were detected in all of the soldiers in the patient group by nested PCR before the treatment, but were not detected in any of the soldiers in control group 1. Among 472 whole blood samples from control group 2 collected at the area of endemicity, \textit{P. vivax} parasites were detected in two soldiers by nested PCR. These soldiers had no clinical signs or symptoms of malaria at the time of our sampling.

\textbf{Recombinant proteins expressing the C-terminal regions of PvMSP1.} The carboxy-terminal 18-kDa region of Pv200 was amplified with primers 6 and 5 under the same PCR conditions mentioned above. The amplified PCR product of 122 bp was excised from an agarose gel and used as a template for renaturation. The renatured DNA was inserted into the expression vector pET-21a and expressed in \textit{Escherichia coli}. The C-terminal region of the recombinant protein was confirmed by Western blotting and amino acid sequence analysis.

\section*{Indirect ELISA.} Serum from each individual was tested for reactivity with the recombinant proteins by indirect ELISA as described in several previous studies (19, 21, 31). In brief, each well of a 96-well enzyme immunoassay plate (Costar, Cambridge, Mass.) was coated with 50 ng of affinity-purified recombinant proteins diluted in phosphate-buffered saline (PBS) (pH 7.4), incubated overnight at 4°C, and then washed three times with PBS-Tween. The plates were blocked at 37°C for 1 h with 5% normal goat serum (Sigma, St. Louis, Mo.) in PBS-Tween. Serum samples were added to duplicate wells at a 1:200 dilution. After 1 h of incubation at 37°C, unbound material was washed away, and peroxidase-conjugated goat anti-human IgG or IgM (Sigma), diluted to 1:40,000, was added to each well. After another hour of incubation at 37°C, the excess labeled antibody was washed away, and a reaction was developed using the o-phenylene diamine (Sigma) substrate system. The plates were read at 490 nm on a Titertek Multiskan Plus MK II ELISA reader (Labsystems, Lugano, Switzerland).

All optical densities at 490 nm (OD\textsubscript{490} values) represented the binding of either IgG or IgM to the recombinant protein after subtracting the background values of the same serum to PBS alone. Each serum was tested in duplicate, and the OD\textsubscript{490} values were averaged. To overcome the differences between plates, all OD\textsubscript{490} values were converted into correction values. The serum of the soldier from the patient group who had one of the highest values of IgG and IgM was selected and added to duplicate wells of all of the tested plates as a positive control. The average OD\textsubscript{490} of the positive control was converted to 2, and the average OD\textsubscript{490} values of all of the tested samples were calculated as follows:

$$\text{Average OD}_{490} \text{ of tested sample} = \text{Average OD}_{490} \text{ of PBS only} \times 2$$

\section*{Construction of expression vector pYLJ-MSP.} The construction of the pYLJ-MSP is shown schematically in Fig. 1. Genomic malaria DNA was prepared from the blood of a patient with Korean vivax malaria. The patient was a soldier serving at Yeoncheon (the northern part of the Kyonggi Province), which has been one of the most prevalent areas for vivax malaria, in the summer of 1998. Using genomic malaria DNA from the patient as a template, the DNA sequence encoding amino acids Asn\textsubscript{122}-Ser\textsubscript{179} was amplified by PCR. The first PCR was done using primers 1 and 3, and the second PCR was done using primers 2 and 3 (Table 1). After initial denaturation (30 s at 94°C), 36 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) were performed. To create pBc-Pv200-ct657, the amplified DNA was ligated into an EcoRV-digested pbLueScript KS(−) vector. pBc-Pv200-ct657 was amplified using PCR with primers 4 and 5 under the same conditions mentioned above. The alpha interferon-pLBC, including the yeast alpha leader sequence (\textit{S. cerevisiae} containing alpha interferon-pLBC, available from the Korean Collection for Type Cultures; accession number KCTC0051BP), was amplified with primers 6 and 7 under the same PCR conditions. To create alpha-Pv200-19, the two amplified DNAs were linked with primers 6 and 5 by overlapping PCR under the same PCR conditions. pYES-2 vector and alpha-Pv200-19 were ligated to create pYLJ-MSP after digestion with HindIII and XhoI. pYLJ-MSP was transformed into the \textit{S. cerevisiae} strain INVSc1. The transformants were propagated and induced as previously described (15).
contains two epidermal growth factor-like domains. The recombinant protein encoding amino acids of this region, Asn \textsubscript{1622}-Ser\textsubscript{1729}, were expressed. Coomassie-stained sodium dodecyl sulfate-polyacrylamide gels showed that this recombinant protein had a molecular weight of 18 kDa and that the protein was reactive with the serum of individuals with patent malaria on Western blot analysis (data not shown).

Anti-Pv200\textsubscript{18} antibody levels in the malaria-naïve control groups and \textit{P. vivax} malaria patients. The anti-Pv200\textsubscript{18} antibody levels are shown in Fig. 2. In control group 1, the mean value and standard deviation (SD) of the IgG levels were 0.053 and 0.029, respectively, and those of the IgM levels were 0.040 and 0.010, respectively. In control group 2, the mean value and SD of the IgG levels were 0.050 and 0.027, respectively, and those of the IgM levels were 0.039 and 0.010, respectively. In the patient group, the mean value and SD of the IgG levels were 1.31 and 0.75, respectively, and those of the IgM levels were 1.03 and 0.62, respectively.

![Diagram of pYLJ-MSP construction](http://cvl.asm.org/)

**FIG. 1.** Construction of pYLJ-MSP. Two amplified DNAs of \textit{P. vivax} and the alpha-factor leader sequence were linked by overlapping PCR. The alpha leader-MSP1 and pYES2 were digested with HindIII and XhoI and ligated to create pYLJ-MSP.
When the cutoff values for seropositivity were determined as mean $\pm 3 \times SD$ of the antibody levels in control group 1 (i.e., $0.14$ for IgG and $0.07$ for IgM), both IgG and IgM antibody levels were below the cutoff values in $98.5\%$ (465 of 472) of control group 2. The IgG and IgM antibody levels were positive in $88.1\%$ (371 of 421) and $94.5\%$ (398 of 421) of the patient group, respectively. Both IgG and IgM antibody levels were below the cutoff values in $12$ subjects from the patient group. Six of these subjects were retested 1 week after the first test, and their antibody levels had converted to seropositive. However, in all six of these subjects the levels of IgG antibody were below $0.48$, and their IgM antibody levels were below $0.13$ (Fig. 3).

In the two patients with recurrent malaria, the IgG levels were both $2.30$. This was the second highest value in the patient group. Their IgM levels were $0.06$ and $0.045$, respectively.

**Longevity of anti-Pv200 antibody responses.** The mean value of the IgG levels of $20$ tested patients had decreased to near the cutoff value by $10$ months after the treatment (Fig. 4a). Antibody levels of IgG started to be converted to seronegative in $4$ patients $6$ months after the treatment, and the total number of seronegatives increased to $6$ patients $7$ months after the treatment, and to $11$ patients $10$ months after the treatment.

**DISCUSSION**

In the present study, we expressed the C-terminal region of the PvMSP1 and evaluated the humoral immune response to this antigen by indirect ELISA. Recombinant Pv20018 had a molecular mass of $18$ kDa, and it had the same amino acid sequence as the Sal-I strain in this region (13). Out of the various antigens to *P. vivax*, the antibody response against the circumsporozoite protein has been the most intensively studied. Previous studies have demonstrated that the levels and frequency of antibodies against the circumsporozoite protein were higher in individuals in areas of endemicity than in those in areas of nonendemicity (28, 31). However, the circumsporozoite protein has been found to have low immunogenicity (17), and more than $20\%$ of the patients in one study did not have detectable antibody levels against circumsporozoite protein at the early stages of symptom development (10).

One previous study demonstrated that PvMSP1 is expressed on the surface of almost all parasites in the erythrocytic stage (16). The mean parasite count in Korean vivax malaria patients with patent malaria infection has been measured at approximately $5,000/\mu l$ (14). This results in sufficient immune recruitment by PvMSP1 in individuals with patent malaria infection. Therefore, we chose PvMSP1 instead of the circumsporozoite as our candidate antigen for the serodiagnosis of patent malaria infection.

In this study, the sensitivity of the test for IgG against PvMSP was higher (88.1%) than in previous studies, which have reported sensitivity levels of 50 to 60% (29, 30).
major difference between our study and the previous ones is the system employed for the expression of PvMSP1. Yeast cells were used for the expression system in our study, whereas an *E. coli* system was employed in the previous studies. Glycosylation processes, which play an important role in creating the three-dimensional conformation of glycoproteins, do not occur in *E. coli* cells. However, they do occur effectively in yeast cells (12). Because PvMSP1 is a glycoprotein, the yeast expression system may be more effective in mimicking PvMSP1 than the *E. coli* expression system. This difference may explain the improved sensitivity in our study.

When mean ± 2 × SD of the antibody levels in control group 1 was regarded as the cutoff value for positive reactions (i.e., 0.11 for IgG and 0.06 for IgM), the sensitivity of the test was 97.9% (412 of 421), and the specificity was 96.4% (455 of 472) in control group 2. When mean + 3 × SD of the antibody levels in control group 1 was regarded as the cutoff value for positive reactions (0.14 for IgG and 0.07 for IgM), the sensitivity of the test was 97.1% (409 of 421), and the specificity was 98.5% (465 of 472) in control groups 2. Therefore, we chose mean + 3 × SD of the antibody levels in control group 1 as the cutoff value for seropositivity.

Of the 472 healthy soldiers in the areas of endemicity, two (0.4%) had asymptomatic parasitemia, which was detected by PCR. Anti-Pv200 antibody levels were significantly raised in both of them (i.e., IgG of 1.2 and 0.8, and IgM of 0.8 and 0.9, respectively).

In this study, the levels and frequency of antibodies against the circumsporozoite protein were higher in malaria-naïve individuals in areas of endemicity than in malaria-naïve individuals in areas of nonendemicity (28, 31). These findings suggest that PvMSP1 may not be a useful antigen in detecting individuals who have been infected with sporozoites but have not yet passed into the erythrocytic stage. In order to detect these individuals, the antigenicity must come into being during the prehepatic stage and must be maintained into the erythrocytic stage. However, PvMSP1 is expressed only during the erythrocytic stage (20), and thus the specific immune response was not available to differentiate the individuals in the areas of endemicity from those in the areas of nonendemicity.

Of note is the fact that the IgG antibody was positive as early as 3 days after the onset of symptoms. In many infectious diseases, it can take 2 to 4 weeks before the seroconversion of the IgG antibody occurs. This strongly suggests that patients have parasitemia long before they develop symptoms. Indeed, the period of asymptomatic parasitemia (prepatent parasitemia plus patent parasitemia) of vivax malaria is known to be 6 to 10 days. The six patients in whom IgG levels were seroconverted had very low IgM levels (Fig. 3). Two explanations may be possible for the low IgM levels. Firstly, because of the affinity maturation during the development of IgG, IgG antibody may have higher affinity to the MSP1 antigen than IgM antibody. Secondly, IgG levels as well as IgM levels were low in the six patients, and this suggests that they might be poor responders to the MSP1 antigen.

The longevity of IgG was more than 1 year in one-third of our subjects, and the longevity of IgM was more than 3 months in most of them. Therefore, we suggest that caution be used when interpreting a positive antibody test.
In the ROK, one-third of all blood donors had traditionally been soldiers. However, since the vivax malaria epidemic began, the number of soldiers donating blood has sharply decreased, because more than half of all of the vivax malaria cases occur in soldiers. A microscopic examination of peripheral blood smears is the standard procedure for the diagnosis of malaria. However, microscopic examination is highly time-consuming and labor-intensive. Therefore, it is not a practical method for mass testing, such as screening donated blood. For this purpose, antibody testing using an immunoassay may be a more useful and efficient method.

In conclusion, PvMSP1 is an adequate antigen for the sero-diagnosis of patent malaria infection, since sufficient amounts of antibody are induced in almost all individuals with patent malaria infection at an early stage of symptom development.

**ACKNOWLEDGMENTS**

We thank the soldiers of the ROK Army who voluntarily participated in this study and their medical and executive officers. We also thank Young-Hoon Kim for his critical review of the manuscript.

This work was supported in part by a Korean Military Medical Association grant for the fiscal year 1999 and by a grant (03-99-022) from the Seoul National University Hospital Research Fund.
REFERENCES


