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Isolation and Serological Characterization of a *Plasmodium vivax* Recombinant Antigen

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A genomic library for *Plasmodium vivax* was constructed in lambda gt11 and immunologically screened with pooled serum samples from vivax patients. Six seroreactive clones were isolated, and one clone, denoted PV9, was studied further. This clone has an unusual base composition (65% G+C), does not share any homology with *P. falciparum*, and codes for an entirely new antigenic determinant. Antibodies (immunoglobulin G type) against the PV9-encoded polypeptide were produced in all vivax patients older than 15 years. This seroreactivity was lower among patients younger than 15 years (53%). The antigenic epitope(s) of the PV9-encoded polypeptide was recognized at a similar rate by serum samples from *P. vivax* patients who were living 350 to 973 km apart. Fifty percent of uninfected Indian adults were also seropositive, whereas all European and American (United States) sera tested were negative, suggesting that anti-PV9 antibodies persist after infection. The seroreactivity pattern of this antigen is similar to that of the immunity developed in malaria after repeated infections.

Malaria remains uncontrolled despite immense efforts being made to handle the disease from various directions (14, 21, 25, 31, 32). The major reasons are vector resistance, operational failures, and emerging drug resistance in *Plasmodium falciparum*. In recent years, this has led to an emphasis on other methods of malaria control such as developing a recombinant malaria vaccine (2, 4, 8, 17, 19, 24, 27). The main work has been centered around *P. falciparum*, in which a large number of antigens have been defined by gene cloning. Some of these antigens could provide protective immunity and thus have been advocated for the proposed vaccine. However, for *Plasmodium vivax*, such progress has been painfully slow, although this is the most common human malaria parasite and affects large numbers of people in tropical countries. The main reason for the slow progress is probably because this parasite, unlike *P. falciparum*, is not yet cultivatable. Its antigens and antigenic determinants need to be studied in depth in order to design the subunit vaccine as well as for seroepidemiology after vaccine trials. So far, to the best of our knowledge, only two antigen genes, one duffy receptor and one rRNA gene, from this parasite have been studied in detail (1, 5, 6, 16, 29). We report here the isolation and serological characterization of a new and unique genomic clone from a *P. vivax* library. The clone expresses an important antigenic determinant which generates an immune response in humans.

MATERIALS AND METHODS

Subjects. Individuals suffering from fever attended malaria clinics at Shahjahanpur and Shankargarh (both in Uttar Pradesh) and Delhi, India. Their blood was examined for the presence of malarial parasites by Giemsa-stained thick and thin films under the light microscope. *P. vivax*-positive patients were chosen, from whom heparinized blood was collected with their full consent. Uninfected blood samples were collected from laboratory personnel and from foreign visitors with their consent. Plasma was separated and stored in aliquots at −20°C. The parasitized erythrocytes were purified by running them through a percoll gradient after they were passed through a Whatman CF11 column (10).

Construction of a genomic library in lambda gt11. Most of the manipulations to construct the library were done according to published protocols (9, 22) as well as by following the manufacturer's instructions wherever applicable. Briefly, the *P. vivax*-infected erythrocytes were pooled from a large number of samples and lysed with saponin, and parasite DNA was isolated as described earlier (12). DNA was digested completely with *Sau*3AI to generate small fragments. The ends of these *Sau*3AI-generated fragments were filled in with the Klenow fragment. Internal *Eco*RI sites were protected before adding *Eco*RI linkers. DNA containing *Eco*RI ends was then ligated to the lambda gt11 dephosphorylated *Eco*RI arms (Promega Biotec, Madison, Wis.) at 15°C overnight. The ligated material was packaged in vitro for 2 h at 22°C by using the packaging extracts and the protocol from Promega.

Immunological screening of the library. The library was plated out at a density of 1,800 bacteriophage particles per 90-mm plate, using *Escherichia coli* Y1090 for immunological screening (9). The nitrocellulose filters to which the expressed proteins were transferred were blocked with 2% bovine serum albumin and 20% fetal calf serum in tris-buffered saline containing 15 mM sodium azide for 2 h at room temperature. The filters were then exposed to pooled antisera (1:150 dilution) obtained from *P. vivax* patients for 4 h at room temperature. The serum pool was preabsorbed with a lysate of *E. coli* to remove anti-*E. coli* antibodies to reduce the background (9). The second antibody, alkaline phosphatase-conjugated anti-human immunoglobulin G (Dakopatts, Glostrup, Denmark), was used at a dilution of 1:750. The antigen-antibody complexes were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Plaques which gave purple spots on the nitrocellulose filter were picked and rescreened at a lower density.

Immunoblot of recombinant antigens. The phage particles from positive clones were lysogenized in *E. coli* Y1089 to prepare the recombinant antigen (9). Each clone was grown

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in liquid culture and induced with isopropyl-β-D-thiogalactopyranoside. Cells were harvested and suspended in 0.05 volume of phosphate-buffered saline containing 1 mM each EDTA, β-mercaptoethanol, phenylmethylsulfonyl fluoride, and dithiothreitol. They were frozen at −70°C overnight and then lysed by sonication. The lambda gt11 nonrecombinant lysogen was also processed and used as a negative control. Lysates from all these clones were also made in exactly the same way except that the cultures were not exposed to isopropyl-β-D-thiogalactopyranoside and were used as uninduced controls. An equal amount of protein (50 μg) of each clone was loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels for separation of protein bands. These bands were transferred electrophoretically onto nitrocellulose filters. The blots were developed by using pooled and preabsorbed serum samples from vivax patients as described above.

Nucleotide sequencing. The recombinant lambda DNA from one of the positive clones (designated PV9) was isolated from cesium chloride-purified phage. The insert was subcloned in M13mp18, and the nucleotide sequence was determined by the dideoxy-chain termination method of Sanger et al. (23) with M13 universal primers as described earlier (26). Sequencing was confirmed from two clones in both directions.

Dot hybridization. The parasite DNA from *P. falciparum* and *P. vivax* was sonicated in a sonication water bath to generate smaller fragments (11). The DNA was then denatured and spotted onto a nitrocellulose filter by using a blotting apparatus (S & S Co., Dassel, Federal Republic of Germany). The filter was hybridized with a nick-translated fragment from one of the positive clones (PV9). The hybridization was done at 42°C in the presence of 50% formamide (12).

Serology. Individual serum samples were adsorbed with *E. coli* lysate to remove anti-*E. coli* antibodies to reduce the background (9). The recombinant antigen, prepared for immunoblots, was spotted in various amounts (0.3, 0.6, 1.8, and 2.4 μg) onto nitrocellulose paper strips. Lysates from *E. coli* lysogenized with nonrecombinant lambda gt11 (producing β-galactosidase) and *E. coli* only were used as negative controls. The filters were developed with serum samples from patients as described above. Each serum sample was tested at four different dilutions (1:50, 1:100, 1:200, and 1:400).

Nucleotide sequence accession number. The PV9 sequence was assigned EMBL accession no. X 53680.

RESULTS

Isolation and characterization of genomic clones. We constructed a *P. vivax* genomic library in the expression vector lambda gt11. About 50,000 clones from this library were screened with pooled serum samples from *P. vivax* patients. In the first cycle of screening, 19 tentatively positive clones were identified. These clones were further screened at a lower density to confirm their positivity. Only six clones were found to be definitely positive.

All six confirmed positive clones were lysogenized and processed for Western immunoblot analysis. The results of this analysis are depicted in Table 1, in which five clones, PV9, PV12, PV16, PV17, and PV19, show fusion protein bands in the range of 125 to 130 kDa. That the bands were of fusion proteins was confirmed by the absence of any such bands in the uninduced cultures. However, no band was seen on Western immunoblots for PV14 from either induced or uninduced cultures. Nevertheless, this clone remained positive on plaques and on dot-enzyme-linked immunosorbent assay (ELISA), indicating that antigenic epitope(s) of the PV14-encoded polypeptide may have been disrupted during SDS-polyacrylamide gel electrophoretic analysis.

TABLE 1. Characterization of positive clones obtained from immunoscreening of *P. vivax* genomic library

Clone	Size of fusion protein (kDa)	Hybridizes with PV9[a]	Seroreactivity in vivax patients[b]
PV9	125	+	++++
PV12	125	−	++
PV14	Unstable[c]	−	++
PV16	130	+	+++
PV17	125	−	+
PV19	130	+	++

[a] The PV9 insert was nick translated and hybridized with other positive clones.

[b] These are preliminary screening data for 23 vivax patients. The serum samples were screened against the six positive clones by dot-ELISA. PV9 is the most seroreactive among six clones.

[c] This clone remained positive on plaques and on dot-ELISA but did not show any band on Western immunoblots.

Nucleic acid hybridization analysis showed that clones PV9, PV16, and PV19 are related, as they cross-hybridized with each other (Table 1). The preliminary data from screening serum samples from 23 vivax patients with all six of these clones showed that PV9-encoded polypeptide was recognized by serum samples from the maximum number of patients compared with other clones.

Sequence analysis of PV9. The nucleotide sequence of the PV9 insert (188 bp) is shown in Fig. 1. The base composition of the insert DNA is 65% G+C. The sequence contains some internal nucleotide repeats; the CGCC unit is repeated in several places. However, there are no perfect peptide repeat units (Fig. 1). This amino acid sequence is derived from the insert by considering the fact that it is expressed as a fusion protein.

The data base search for protein and nucleotide homology showed no significant homology with any of the *Plasmodium* genes. No significant amino acid homology (40% and above) was found with other proteins; the maximum homology (32%) was observed with the vitellogenin-2 precursor of *Caenorhabditis elegans*. Pol polyprotein of T-cell leukemia virus (HTLV-II) and immediate-early protein LE 17 of herpes simplex virus showed 31 and 30.4% amino acid sequence homology, respectively, with the PV9-encoded peptide. However, a significant nucleotide homology was observed with several plant genes; the maximum homology (54%) was observed with the *Rhizobium meliloti ntrA* gene encoding NtrA protein.

Specificity of PV9. The PV9 insert was used to hybridize with *P. vivax* and *P. falciparum* DNA. The results are shown in Fig. 2. The PV9 insert showed no hybridization with *P. falciparum* DNA. It only hybridized with *P. vivax* DNA, indicating that it shares no homology with *P. falciparum*. The PV9 insert did not hybridize with human DNA under these conditions (data not shown).

Serology. Sixty-seven serum samples from *P. vivax* patients, 18 samples from uninfected individuals residing in India, and 9 samples from uninfected individuals living abroad (four European and five American [United States]) were tested for the presence of antibodies against the PV9-encoded antigen. The results are shown in Table 2. The total seropositivity rate among *P. vivax* patients was 89%. About

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 Asn Ser Asp Arg Ile Ala Arg Leu Met Ala Leu Ala Pro Gln Pro  15
GAAT TCC GAT CGT ATC GCG CGC CTG ATG GCG TTG GCG CCT CAG CCA  46
 EcoR I  Sau3A I

Ser Gln Ala Leu Pro Tyr Gln Thr Glu Tyr Leu Ala Pro Arg Ser   30
TCG CAG GCC CTG CCG TAC CAA ACG GAA TAT CTC GCG CCA AGG TCG   91
                  Rsa I

Phe Ala Pro Met Arg Pro Pro Arg Arg Gly Leu Ser Gly Leu Trp   45
TTC GCC CCG ATG CGC CCG CCA CGC CGG GGC CTG AGC GGC CTT TGG   136


Arg ***                                                      46
CGC TAAATCACTCGAAAGGAACCCCCGCCATGGCTTACCACGATCGGAATTC        188
           Taq I           Nco I      Sau3A I EcoR I
```

FIG. 1. Nucleotide sequence of PV9 clone. The total insert was subcloned into M13mp18 and sequenced in both directions by the dideoxy-chain termination method. The predicted amino acid sequence is derived from the insert DNA sequence in frame with β-galactosidase. The amino acids and nucleotides derived from the vector are shown in bold letters. The restriction sites are shown and their sequences are underlined.

50% of uninfected Indian serum samples were also positive for anti-PV9 antibodies, but none of the European and American serum samples tested were positive. Some of the serum samples were independently positive for the β-galactosidase of lambda gt11. The anti-PV9 antibody levels and the frequency of seropositivity were lower among children (Table 2). However, all patients from India older than 15 years were seropositive for anti-PV9 antibodies, regardless of their geographical location (Table 3). The total seropositivity rates for serum samples from patients from Delhi, Shahjahanpur, and Shankargarh were similar. It is important to note that these places are several hundred kilometers apart (Table 3). A correlation was observed between anti-PV9 antibody titer and age of the patient (Fig. 3).

DISCUSSION

We isolated a unique *P. vivax* genomic clone, denoted PV9, by immunological screening. The polypeptide encoded by this clone is different from the malarial genes already identified. It does not cross-react with *P. falciparum* as noticed during DNA dot-hybridization experiments (Fig. 2). The base composition of this clone is somewhat unusual, being G+C-rich. However, this could not be considered the final G+C content of the corresponding gene, because PV9 is incomplete at the 5′ end and the nucleotide sequence of the complete gene is yet to be elucidated. The higher G+C content has also been reported in previously cloned genes of *P. vivax*; for example, there is a 47% G+C content in PV200 (1, 5, 16). The *P. vivax* genome has been shown to contain a slightly higher G+C content than that of *P. falciparum* (15, 30). In general, the malarial genome is A+T-rich, with the noncoding regions containing a somewhat higher A+T content than the coding regions (15, 26, 30, 33).

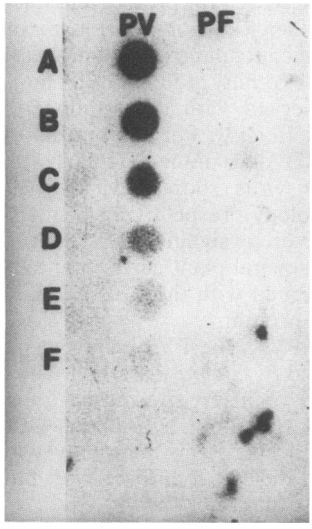

FIG. 2. Dot-blot hybridization of PV9 insert with *P. vivax* and *P. falciparum* DNA. Different amounts of DNA were spotted: A, 2 μg; B, 1 μg; C, 500 ng; D, 250 ng; E, 125 ng; F, 62.5 ng. The PV9 insert was nick translated and hybridized at 42°C in 50% formamide. The filter was finally washed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% SDS at 65°C for 1 h. PV, *P. vivax*; PF, *P. falciparum*.

Antibodies against the PV9-encoded polypeptide are produced in all vivax patients older than 15 years during the natural course of infection. Also, the serum samples from vivax patients from different regions showed seropositivity without any discrimination. This indirectly suggests that there is no strain variation as such with reference to the PV9-encoded antigenic epitope(s). However, the definitive proof requires sequencing data for the PV9 insert from different strains; a similar high frequency of recognition has been reported for fusion proteins obtained from the dimorphic regions of *P. falciparum* gp190 (18). It can be stated here that antigenic polymorphism and strain diversity have been reported in *P. vivax* within Sri Lanka (13, 28). Antigenic variation and strain diversity in *P. falciparum* are already well-established facts which are posing a serious problem in developing a universal malaria vaccine.

The antibodies against PV9 persist in 50% of uninfected individuals from India. Moreover, there is a correlation

TABLE 2. Seroreactivity of PV9-encoded antigen in individuals

Age group (yr)	No. of individuals	No. sero-positive (%)	Avg titer
Vivax patients			
3–15	15	8 (53)	162
16–62	52	52 (100)	365
Total	67	60 (89)	276
Uninfected controls			
Indians (20–45)	18	9 (50)	245
Europeans (25–49)	4	None	ND[a]
Americans (25–50)	5	None	ND

[a] ND, exact titer was not determined, but these samples remained negative at 1:50 dilution.

TABLE 3. Anti-PV9 antibodies in *P. vivax* patients with different geographical origins

Place	Distance from Delhi (km)	No. of patients	No. seropositive (%)[a]	Avg titer
Delhi		23	22 (95)	283
Shahjahanpur	350	25	21 (84)	291
Shankargarh	973	19	17 (89)	217

[a] All adults (older than 15 years) from all these places were seropositive against PV9.

between antibody titer and age of the patient (Fig. 3). The pattern of antibody production against this antigen is somewhat similar to that of the immune response shown by protective antigens of *P. falciparum* (3, 18, 20). It is known that young children are less immune to malarial infections than older people. This may be related to the fact that immunity to malaria is developed after repeated attacks (7).

The genomic clone described here is significant not only because in *P. vivax* merely two antigen genes have previously been cloned and sequenced (an rRNA gene and duffy receptor) but also because (i) it has an unusually high G+C content for a *Plasmodium* gene; (ii) it does not cross-react with *P. falciparum*; (iii) all the adult patients produce antibodies during the natural course of an infection which react with lysates of it; (iv) it has an antigenic epitope(s) which probably is conserved among regional isolates; and (v) the antibody titer against it increases with age. This antigen thus may be involved in providing protection and could serve as a potential candidate for the future malaria vaccine. However, several studies, including cellular location and native size of the antigen, detailed human T-cell studies, complete gene sequence, etc., are required.

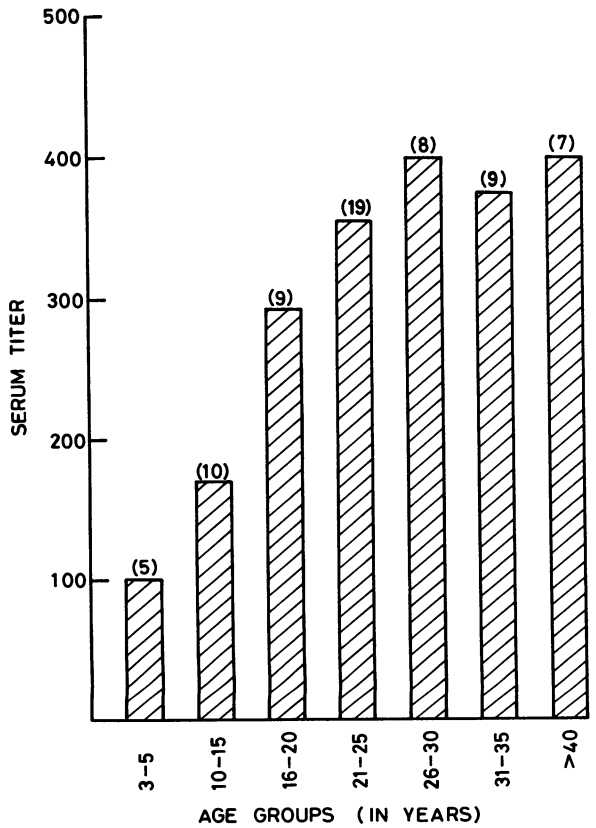

FIG. 3. Antibody levels against PV9-encoded antigen in vivax patients among different age groups. In this study, the age range was from 3 to 62 years. The number of patients tested in each age group is indicated above the bars. Each bar indicates the mean value of the serum titer.

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