Presence of Memory T Cells and Naturally Acquired Antibodies in *Plasmodium vivax* Malaria-Exposed Individuals Against a Group of Tryptophan-Rich Antigens With Conserved Sequences

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**Background.** Tryptophan-rich antigens of malarial parasites have been proposed to be the potential vaccine candidate antigens. *Plasmodium vivax* contains the largest number of such antigens, which need to be evaluated for their immune responses.

**Methods.** Recombinant proteins of 15 *P. vivax* tryptophan-rich antigens (PvTRAgs) were expressed, purified, and used for the human humoral and cellular immune responses. Genetic polymorphism of these 15 genes was also determined among clinical *P. vivax* isolates.

**Results.** The T lymphocytes of *P. vivax* exposed individuals expressed higher level of CD69 against all 15 PvTRAgs. These antigens also activated the large population of CD4+ T cells and produced higher level of intracellular IL-2, INF-γ and IL-4. Although there was a mixed Th1 and Th2 response against these antigens, this response was biased toward Th2. The majority of *P. vivax* patients (75.7%–100%, n = 33) produced IgG antibodies against these antigens. Most of these antigens showed conserved T- and B-cell epitopes in the parasite population.

**Conclusions.** These results suggest the presence of memory T cells in humans against these antigens to generate faster and more specific immune responses to minimize the *P. vivax* infection. Further characterization of these PvTRAgs may lead to the identification of a potential therapeutic target.

**Keywords.** Vivax malaria; Tryptophan-rich antigens; cytokines; humoral and cellular immune responses; CD4+ T cells.

*Plasmodium* induces a specific immune response by stimulating the release of cytokines, which play an important role in activating the host macrophages, neutrophils, T cells and natural killer (NK) cells to react against liver and blood stage parasites [1]. Specific cytokines reported to be of importance in human malaria include tumor necrosis factor α (TNF-α), interferon gamma (IFN-γ), and interleukins (IL-6, IL-10, IL-2, and IL-4) [2]. Several studies have examined the association between various signs and symptoms of *Plasmodium vivax* malaria patients and their serum cytokines levels [3–6].

The results of vaccine trials in the field indicated that both humoral and T-cell–mediated immunity are important for inducing protection against malaria [7]. Such immune responses against several vaccine candidate antigens of *Plasmodium* have been characterized [8–18]. However, all of these antigens show genetic polymorphism that could be one of the problems in vaccine development. Extensive efforts are therefore required to identify the antigens that elicit protective immunity against malaria, yet their sequences are conserved in the parasite population.

Tryptophan-rich proteins have been identified in murine and human malaria parasites [19–26]. Across
the Plasmodium species, these proteins have positionally conserved tryptophan residues. These proteins are highly immunogenic in nature and have been proposed as the potential malaria vaccine candidate antigens [9, 10, 21–23, 25–27]. This is because in a murine malaria model they were able to provide protection against Plasmodium yoelii [9, 10]. Remarkably, large numbers of tryptophan-rich protein coding genes have been identified in the P. vivax genome than in any other malarial parasite species (www.plasmodb.org). These antigens need to be immunologically characterized. Earlier, we have been able to characterize some of them separately and showed that they were able to generate humoral and cellular immune responses in humans [21, 22, 24–27]. These observations warranted us to investigate the comparative immune responses against the group of P. vivax tryptophan-rich antigens (PvTRAGs) together in humans and also study the sequence diversity in T- and B-cell epitopes of these antigens in the parasite population. We therefore used 15 of these PvTRAGs, including those described previously, to study genetic diversity in parasite population, as well as the humoral and cellular immune responses in humans exposed to this parasite. This approach should identify the potential vaccine candidate PvTRAg that elicits the best immune responses in humans yet have the conserved B- and T-cell epitopes. Results showed that P. vivax exposed individuals were able to generate humoral as well as cell-mediated immune responses against these antigens and had conserved B- and T-cell epitopes in parasite population. Intracellular cytokine levels elicited by all 15 PvTRAGs revealed mixed Th1/Th2 response but biased toward Th2.

**METHODS**

**Subjects**

Individuals living in Northern India were screened for the clinical symptoms of malaria and presence of malarial parasite by light microscopy. For genetic polymorphism and serological studies, 200–500 mL of heparinized blood was collected from the microscopically confirmed P. vivax malaria patients prior to start of antimalarial drugs. Patients were treated with antimalarial drugs according to the national drug policy (www.nvbdcd.org). The same amount of heparinized blood was also collected from uninfected healthy individuals from the same area. For cellular immune response studies, 5 mL of heparinized blood was collected from 4 malaria-naive volunteers and 10 individuals who had recovered from their last P. vivax malaria episode about 8–12 weeks prior to sample collection. Samples were stored on ice and transported to the lab within 6 hours. These individuals were informed about the study and had given their informed consent. The study was approved by the ethics committee of the All India Institute of Medical Sciences, India. Institutional ethical guidelines were followed while collecting blood samples from individuals.

**Antigens and Monoclonal Antibodies**

Fifteen different recombinant PvTRAGs were used in this study for their comparative immunological responses (Figure 1). We selected these first 15 of 36 proteins because they were showing higher sequence homology compared to the previously identified PvTRAg [24]. Polymerase chain reaction (PCR) amplification, cloning, and expression and purification of PvTRAg69.4, PvTRAg38, PvTRAg53.7, PvTRAg43.1, PvTRAg42.9, PvTRAg39.9, and PvTRAg32.4 were carried out in the present study. For this purpose, the parasite DNA isolated from the P. vivax patient’s finger prick blood was used for PCR amplification of these genes using primers and conditions as given in Supplementary Table 1. The amplified products for all of these genes were cloned in the expression vector pPROEXHTa except for PvTRAg69.4 and PvTRAg42.9, which were cloned in to pPROEXHTb. Expression of the recombinant His-tagged proteins was induced by adding 1 mM of IPTG and purified by immobilized metal affinity chromatography (IMAC) on Ni²⁺-NTA agarose (Qiagen GmbH, Hilden, Germany). Purified recombinant proteins were analyzed on 12% SDS-PAGE and further confirmed with western blot using anti-His6 monoclonal antibody. Cloning, expression, and purification of PvTRAg, PvTRAg40, PvTARAg55, PvTRAg80.6, PvTRAg39.8, PvATRAg74, PvTRAg35.2, and PvTRAg33.5 have been described elsewhere [21–26].

Monoclonal antibodies; anti-CD3 PEcy5, anti-CD4 APC-Cy7, anti-CD69 FITC, anti-IL-4 FITC, anti-IL-10 PE, anti-INF-γ PECy7, anti-IL-2 APC, and CD28 were obtained from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, California) and used at the manufacturer’s recommended concentrations.

**Cellular Immune Response**

**Cell Preparation and Antigenic Stimulation**

Whole blood (100 µL) was diluted 1:1 in RPMI supplemented with 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen, Carlsbad, California) and distributed into 96-well flat-bottom tissue culture plate (BD Biosciences, San Diego, California). The co-stimulatory monoclonal Ab, CD28, was added to whole blood at a concentration of 5 µg/mL. Cells were stimulated with Ni-NTA affinity chromatography, purified, and filter sterilized recombinant PvTRAGs at a final concentration of 10 µg/mL, or phytohemagglutinin-1 (5 µg/mL), or with media only. The culture tubes were incubated upright in a humidified 37°C, 5% CO₂ incubator for a total of 60 hours. The secretion inhibitor brefeldin A (eBioscience, Inc, San Diego, California) at a 10 µg/mL concentration was included for the final 4 hours of activation.

**Immunolabeling of Cell Surface Antigens and Intracellular Cytokines**

At 64 hours, 100 µL of 20 mM EDTA (2 mM final concentration) was added directly to the whole blood cultures
Figure 1.  Sequence alignment of *Plasmodium vivax* tryptophan-rich proteins. The conserved tryptophan residues are shown in bold and shaded in gray. Asterisks indicate identical amino acids, gaps are indicated with dashes, and double and single dots indicate conserved and semiconserved substitutions, respectively. Numbers on the right indicate amino acid positions. These sequences are retrieved from PlasmoDB database (www.plasmodb.org), and their IDs are as follows: PvTRAg (PVX_090265), PvTRAg9.4 (PVX_115465), PvTRAg38 (PVX_101515), PvTRAg38 (PVX_090260), PvTRAg39 (PVX_090255), PvTRAg39.8 (PVX_088810), PvTRAg35.2 (PVX_109280), PvTRAg35.2 (PVX_121897).

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and incubated for 15 minutes at room temperature followed by simultaneous lysis of erythrocytes and fixation of leukocytes using FACS lysing solution (BDIS, San Jose, California). The cells were then washed with phosphate-buffered saline, bovine serum albumin (0.5%), and sodium azide (0.1%). They were suspended in 400 µL of wash buffer and distributed in equal volumes in 2 sets of staining tubes for immunolabeling.

Monoclonal antibodies (MAbs) against the surface determinants were added to one set of tubes in the following combination: CD3 PECy5, CD4 APC-Cy7, and FITC CD69. The cells were incubated with MABs for 30 minutes at room temperature. After washing they were fixed with 1% paraformaldehyde and kept in darkness at 4°C until acquisition.

In another set of tubes, 2-step immunolabeling was performed. In the first step, MAbs against the surface determinants of CD3 and CD4 were added. The cells were incubated for 30 minutes at room temperature, washed with washing buffer, and resuspended in FACS Permeabilizing Solution 2 (BD Biosciences, BDIS, San Jose, California) for 20 minutes at room temperature. Cells were again washed, and pelleted cells were labeled (30 minutes at room temperature) for intracellular cytokines by using anti-IL-4 FITC, anti-IL-10 PE, anti-INF-γ PECy7, and anti-IL-2 APC MABs. After labeling, cells were washed, fixed with 1% paraformaldehyde, and kept in darkness at 4°C until acquisition.

Flow-Cytometric Acquisition and Analysis
Samples were analyzed in a BDLSRII Flow Cytometer (Becton Dickinson Immunocytometry Systems, Palo Alto, California) by using Facs Diva software. Twenty thousand events were acquired for all tests. Forward scatter vs side scatter gating was employed in the data analysis.

Humoral Immune Response
Antibodies against PvTRAGs in patient serum samples were detected by enzyme-linked immunosorbent assay (ELISA). Purified recombinant PvTRAGs were coated in duplicate (100 ng/well) in a 96-well microtiter plate (BD Biosciences, San Diego, California). ELISA was performed as described elsewhere [24] using serum samples from individuals who had been exposed to P. vivax malaria and those who had no recent history of malaria. Serum samples were used at a dilution of 1:200 and horseradish peroxidase conjugated rabbit anti-human immunoglobulin G (IgG) as a secondary antibody (Thermo Fisher Scientific Inc, Rockford, Illinois) at a dilution of 1:2000. Optical density (OD) was measured at 595 nm in a microplate ELISA reader (Bio-Rad Laboratories Inc, Hercules, California). The final reading of each well was obtained by subtracting the OD of the control wells, where no antigen, no primary antibody, no secondary antibody, and no substrate was added.

Genetic Polymorphism
The parasite DNA from clinical isolates was used to amplify by PCR the same region of each of the 15 Pvtrag genes, which was used for cloning and expression purpose (as described above). These PCR products were gel purified using the Gel Extraction Kit (Bioneer Corp, Korea), in accordance with the manufacturer’s instructions and subjected to nucleotide sequencing. Cycling parameters of sequencing PCR and cleanup protocols for sequencing were the same as described elsewhere [28]. The products were sequenced from both strands using primers described in Supplementary Table 2 and ABI Big Dye Terminator Ready Reaction Kit version 3.1 on an ABI Prism 3130xl Genetic Analyzer (PE Applied Biosystems, California). The BioEdit Sequence Alignment Editor and GeneDoc version 2.6.002 were used to analyze the sequencing electropherograms and generate sequence alignment, respectively. All of the sequences generated here have been submitted to the NCBI GenBank database under the accession numbers described in Table 1.

Statistical Analysis
Statistical analysis was performed with SPSS software (ver. 13). Descriptive data were expressed as the mean ± standard deviation or mean ± standard error of the mean for nonparametric distributions. Differences in the levels of antibody and cytokines between groups were compared by using an unpaired Student t-test. A P value ≤ .05 was considered significant.

Results
Activation of CD69 Expression by PvTRAGs
Expression of CD69 in response to different stimuli (PvTRAGs) was analyzed in whole blood samples using flow cytometry. The frequency of CD69+ cells was significantly higher (P < .05) against all 15 PvTRAGs among P. vivax exposed individuals than the malaria-naive individuals (Figure 2). However, there was no significant difference in the number of CD69+ cells among P. vivax exposed and naive individuals if the cells were stimulated with phytohemagglutinin. Pattern of CD69 expression in patients against different antigens was more or less similar, varying from 19.23% to 31.4% (Figure 2). The relative levels of CD69 expression in patients’ blood, expressed as mean fluorescence index, are given in Supplementary Table 3. These index values increased several times after stimulation with PvTRAGs.

Expression Pattern of CD69 Among CD3+ and CD3+CD4+ Cells Against PvTRAGs
Induction of CD69 expression on CD3 and CD4 positive T cells of P. vivax exposed individuals was analyzed to compare the antigen specific activation. Expression of CD69 was almost similar in both CD3+ and CD4+ cells: 54%–72%
Table 1. Genetic Polymorphism in *Plasmodium vivax* Tryptophan-Rich Antigens Among Clinical Isolates

<table>
<thead>
<tr>
<th>Antigen (n)</th>
<th>Region Analyzed (aa position)</th>
<th>Haplotypes (% Prevalence)</th>
<th>Positions of Amino Acid Substitutions(^a)</th>
<th>Accession Numbers (References)</th>
</tr>
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<tbody>
<tr>
<td>PvTRAg32.4  (n = 31)</td>
<td>1–262</td>
<td>HI (100)</td>
<td>Same as Salvador-1 strain type</td>
<td>EF547859–EF547889 (this study)</td>
</tr>
<tr>
<td>PvTRAg33.5  (n = 31)</td>
<td>24–268</td>
<td>HI (100)</td>
<td>Same as Salvador-1 strain type</td>
<td>EU529797–EU529826 (this study)</td>
</tr>
<tr>
<td>PvTRAg35.2  (n = 33)</td>
<td>24–274</td>
<td>HI (100)</td>
<td>Same as Salvador-1 strain type</td>
<td>GU229707–GU229738 [21]</td>
</tr>
<tr>
<td>PvTRAg38    (n = 31)</td>
<td>58–316</td>
<td>HI (70.9)</td>
<td>Q200P</td>
<td>JQ321361–JQ321364 (this study)</td>
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<tr>
<td></td>
<td></td>
<td>HII (29.0)</td>
<td>Q200P, V270E</td>
<td></td>
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<tr>
<td>PvTRAg39.8  (n = 31)</td>
<td>1–322</td>
<td>HI (96.7)</td>
<td>Same as Salvador-1 strain type</td>
<td>EU446027–EU446057 [22]</td>
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<td></td>
<td></td>
<td>HII (32.2)</td>
<td>S32R</td>
<td></td>
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<tr>
<td>PvTRAg39.9  (n = 37)</td>
<td>60–326</td>
<td>HI (21.6)</td>
<td>Same as Salvador-1 strain type</td>
<td>JQ321370, JQ321348–JQ321350 (this study)</td>
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<td></td>
<td></td>
<td>HII (27)</td>
<td>V153F</td>
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<td>HIII (35.1)</td>
<td>N311K</td>
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<td></td>
<td>HIV (16.2)</td>
<td>V153F &amp; N311K</td>
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<td>EF472686–EF472720 [25]</td>
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<td>61–346</td>
<td>HI (100)</td>
<td>Same as Salvador-1 strain type</td>
<td>JQ321365 (this study)</td>
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<td>HII (32.2)</td>
<td>S32R</td>
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<td>HIII (27)</td>
<td>V153F</td>
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<td>HIV (16.2)</td>
<td>V153F &amp; N311K</td>
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<td>PvTRAg53.7  (n = 32)</td>
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<td>HI (100)</td>
<td>Same as Salvador-1 strain type</td>
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<td>PvTRAg55    (n = 31)</td>
<td>94–478</td>
<td>HI (83.3)</td>
<td>Same as Salvador-1 strain type</td>
<td>EF547891–EF547921 [26]</td>
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<td>Insertion of heptapeptide (GVAAPG) at aa position 331</td>
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<td>HII (3.2)</td>
<td>P411S, A413 T, Deletion of ‘A’ at aa position 414 Deletion of heptapeptide (EETAASS) at 420–426 aa, T428A, T430P</td>
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<tr>
<td>PvTRAg69.4  (n = 32)</td>
<td>322–588</td>
<td>HI (56.2)</td>
<td>Same as Salvador-1 strain type</td>
<td>JQ321368–JQ321369 (this study)</td>
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<td></td>
<td></td>
<td>HII (43.7)</td>
<td>N340S</td>
<td></td>
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</table>

\(^a\) Accession Numbers (References)
among CD3⁺ cells and 41%–60% among CD4⁺ T cells (Figure 3). Maximum expression of CD69 among CD3⁺ cells was shown by PvTRAg32.4 followed by PvATRAg74, whereas its expression among CD4⁺ T cells was best shown by PvTRAg53.7 followed by PvTRAg40 and PvATRAg74. It seems that some of the PvTRAgs were inducing CD69 expression to the same level in both CD3⁺ as well as CD4⁺ T cells, whereas others were following a different pattern.

### Intracellular Cytokine Levels in CD3⁺CD4⁺ Cells

The frequency of cytokine-expressing CD3⁺CD4⁺ cells in response to different PvTRAgs was compared between malaria-naive and *P. vivax* exposed individuals (Table 2). The number of cells that produced IL-4 was significantly higher (*P < .05*) among *P. vivax* exposed individuals than healthy controls against most of the PvTRAgs (except PvATRAg74, PvTRAg38, PvTRAg80.6, PvTRAg32.4, and PvTRAg33.5 among *P. vivax* exposed individuals than the healthy controls. The mean percentage of IFN-γ producing CD3⁺CD4⁺ cells was significantly higher (*P < .05*) among *P. vivax* exposed individuals than the healthy controls against 13 of 15 PvTRAgs except PvTRAg32.4 and PvTRAg33.5. In summary, the CD3⁺CD4⁺ cells of *P. vivax* exposed individuals were producing higher levels of IL-2, IL-4, and IFN-γ than IL-10 against most of these PvTRAgs (Table 2).

### Human Humoral Response Against PvTRAgs

Antibody (IgG) levels against purified recombinant PvTRAgs were analyzed in serum samples from 33 *P. vivax* exposed individuals and 30 healthy volunteers who had no recent history of malaria infection. Seropositivity rates and antibody levels varied from antigen to antigen. Seropositivity rate (showing OD at 495 nm more than the cutoff value, ie, mean ±2 SD of healthy controls) varied from 75.76% (for PvTRAg69.4 and PvTRAg33.5) to 100% (for PvTRAg942.9) to 100% (for PvTRAg80.6) (Figure 4). Antibody levels among *P. vivax* exposed individuals were highest
against PvTRAg33.5 compared to others. These results thus show that PvTRAg33.5 produces higher humoral immune response than other PvTRAgs among \textit{P. vivax} exposed individuals (Figure 4). In previous studies, some of these antigens (PvTRAg, PvTRAg35.2, PvTRAg39.8, PvTRAg40, PvTARAg55, PvATRAg74, and PvTRAg80.6) were individually studied for the presence of naturally acquired antibodies in \textit{P. vivax} malaria patients [21–26]. Seropositivity rate for these antigens among \textit{P. vivax} malaria-exposed individuals was found to vary from 82% to 100%, which is somewhat similar to that of the results obtained here.

Genetic Polymorphism

A total of 58 \textit{P. vivax} clinical isolates were analyzed for the genetic polymorphism study. Among them, some isolates failed to amplify all 15 PvTRAgs. The sequence polymorphism data for all of these antigen genes are shown in Table 1. Results showed that 6 of these 15 antigens (PvTRAg 32.4, PvTRAg33.5, PvTRAg35.2, PvTRAg40, PvTRAg42.9, and PvTRAg53.7) were highly conserved in the parasite population as they did not show any sequence diversity among isolates. Four other antigens (PvTRAg38, PvTRAg39.8, PvTRAg69.4, and PvTRAg80.6) also showed very little variation as there were only 2 haplotypes among the isolates for each antigen. Antigen PvTRAg, PvTRAg39.9, and PVTARAg55 had 4 haplotypes each in the parasite population. The highest rate of variation was observed for PvTRAg43.1 (9 haplotypes) and PvATRAg74 (14 haplotypes) among isolates, but the tryptophan-rich region of these antigens were conserved.

DISCUSSION

We describe here the immune responses in humans against a group of PvTRAgs. Earlier we have described immune responses against some of these PvTRAgs but separately for each antigen [21–26]. This is probably the first time to our knowledge that immune responses are described against such a large number of antigens together for a comparative analysis. Almost all of the 15 PvTRAgs were able to generate the humoral and cellular immune responses among \textit{P. vivax} exposed individuals. The B- and T-cell epitopes of these antigens were highly conserved in the parasite population. Expression of significantly higher level of CD69 on leucocytes of \textit{P. vivax} exposed individuals than healthy malaria-naive controls (Figure 1) suggests that all 15 PvTRAgs have the ability to activate T lymphocytes. Results also indicate that there are memory T cells present in the malaria-exposed individuals against these antigens, which were activated faster in our experiments than those from malaria-naive individuals. It is known that memory T cells get activated faster than the naive T cells on secondary infection. This prevents or reduces the
renewed occurrence of disease symptoms [29]. Our results therefore provide evidence for the presence of memory T cells against PvTRAGs in *P. vivax* exposed individuals. However, the expression pattern of CD69 among CD3+ and CD4+ cells (Figure 3) indicates that, in addition to T cells, some other cells like NK cells and B cells may also be expressing this activation marker but to a lesser extent [30].

Cellular immunity is characterized by the expression of cytokines in mature T lymphocytes against infectious agents [31–34]. Therefore, clinical expression, severity, and outcome

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**Figure 3.** Expression of CD69 among CD3+ (A) and CD3+CD4+ (B) cells of *P. vivax* exposed individuals against *P. vivax* tryptophan-rich antigens (PvTRAGs). Each bar indicates the mean percentage ± SEM.
of any infectious disease can be determined by cytokine profile [35]. Cytokines act as regulatory proteins of immune systems that directly or indirectly inhibit the pathogenesis of infectious diseases. Cytokines produced by CD4+ T cells against Plasmodium specific antigens have provided protection against malaria [36–38]. The pattern of cytokine expression by CD4+ T cells of malaria-exposed individuals after stimulation with PvTRAs observed here was strongly dominated by IL-4 followed by IL-2 and IFN-γ. Because the phenotype of CD4+ T cells can be determined by the cytokines expressed by them (IFN-γ and IL-2 for Th1 response and IL-4 and IL-10 for Th2 response), the results indicate that PvTRAs were producing mixed Th1 and Th2 type response. However, the geometric mean ratio of IFN-γ to IL-4 being <1 is an indication of predominance of Th2 response (Supplementary Table 3).

In our previous studies, the level of extracellular cytokines was measured in response to some of these antigens, where IL-10 was produced at the highest level and IFN-γ at the lowest [21–23, 25–27]. On the contrary, the percent of intracellular IL-10 and not IFN-γ producing T cells was lowest in the present study. This difference between intracellular and extracellular cytokine production could arise if IFN-γ is secreted by T cells only, whereas IL-10 is secreted by cells other than T cells as well. Production of higher levels of intracellular and extracellular IL-4 against these antigens in both studies indicates that this cytokine is being produced mainly by T cells in addition to some other cells. Alternatively, this difference in intracellular and extracellular levels of certain cytokines could also arise due to their different secretory nature. Despite the difference in intracellular and extracellular cytokines levels, the geometric mean ratio of IFN-γ to IL-4 was <1 in both studies, which is an indication of Th2 biased response.

Antibody-dependent mechanisms play an important role in reduction of parasitemia and can diminish clinical symptoms in humans [39, 40]. Malaria-exposed individuals mount an antibody response to several antigens present on sporozoites, merozoites, and on infected erythrocytes. Naturally acquired antibodies observed here against these PvTRAs in the patients with high seropositivity rates (75%–100%) also hold significance in this regard. Most of the PvTRAs were producing higher levels of IL-4 and naturally acquired antibodies. This is similar to the reports that a relationship exists between the activation of IL-4 producing T-cell subsets and antibody production in human system in which the immune response is induced by natural infection [41].
Antigenic variation plays an important role in immune evasion, and most of the malarial vaccine candidates have shown such variation in the parasite population. This has hampered the development of a universal malaria vaccine. Keeping this in mind, we investigated the sequence variation for these PvTRAgS in the P. vivax parasite population. This was important as these PvTRAgS show good cellular and humoral immune responses among P. vivax exposed individuals. P. vivax parasite population was found to contain the conserved sequences for majority of PvTRAgS except fewer of them (Table 1). The region of each of the expressed recombinant PvTRAg used for cellular and humoral immune responses is also covered here for the genetic polymorphism studies. Therefore, the results of the genetic polymorphism indicate that the T- and B-cell epitopes of PvTRAgS were highly conserved in the parasite population. Such conserved T- and B-cell epitopes are a desirable feature of a malaria vaccine candidate antigen. Additional studies are required to investigate the functional significance of these PvTRAgS in host-parasite interaction, which could be used to raise the therapeutic reagents to control the disease. Our unpublished results have shown that 6 of these 15 antigens showed erythrocyte binding activity (R. K. Tyagi and Y. D. Sharma, unpublished data) and thus could be of importance for parasite survival and growth in the host. Therefore, on the basis of the above mentioned facts, these PvTRAgS should be investigated further to select a potential vaccine candidate antigen for P. vivax malaria. Furthermore, their cross-reactivity with P. falciparum orthologues should also be investigated.

Figure 4. Naturally acquired antibody levels against different Plasmodium vivax tryptophan-rich antigens (PvTRAgS) among P. vivax exposed individuals. ELISA was performed using serum samples at a 1:200 dilution from P. vivax exposed individuals and malaria-naive individuals against different PvTRAgS. The solid horizontal lines indicate the mean optical density (OD) values and the dashed horizontal line indicates the cutoff values. Solid circles represent the OD values for P. vivax exposed subjects, and open circles represent values for healthy uninfected individuals.

Supplementary Data

Supplementary materials are available at the Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We are grateful to all patients who donated their blood samples for this study. We are grateful to Dr H. K. Prasad and Dr S. N. Das for critical evaluation of the manuscript. We also acknowledge the support provided by the Biotechnology Information System (BTIS) of the department, and Shalini Narang for preparing this manuscript.

Financial support. Indian Council of Medical Research (grant 75/23/2000/-ECIDII to Y. D. S.); Department of Biotechnology (grant BT/PR9800/MED/29/44/2007 to Y. D. S. and Senior Research Fellowships to M. Z. and H. B.), Government of India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Potential conflicts of interests. All authors: No reported conflicts.

The authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References


