DEMONSTRATION OF ANTI-DISEASE IMMUNITY TO PLASMODIUM VIVAX MALARIA IN SRI LANKA USING A QUANTITATIVE METHOD TO ASSESS CLINICAL DISEASE

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Abstract. Clinical immunity to malaria was studied by quantifying the intensity of symptoms as well as by measurement of several hematologic indicators of pathology (the erythrocyte sedimentation rate [ESR], serum bilirubin, reticulocyte count, plasma tumor necrosis factor-α [TNF-α], and blood glucose levels) in 39 Plasmodium vivax malaria patients exposed to endemic malaria in southern Sri Lanka, and for comparison in 43 nonimmune patients who were residents of nonmalarious regions of the country. The intensity of 11 symptoms was scored numerically in all patients using a questionnaire. This clinical score was validated by introducing internal controls to the questionnaire, and by correlating it with the underlying pathology. Both the intensity of clinical disease as well as the degree of underlying pathology were found to be significantly lower in endemic area patients (mean clinical score = 8.8, median ESR = 8 mm) compared with the nonendemic area patients (mean clinical score = 19.0, median ESR 31.5 mm). Endemic area patients also had lower parasite densities (mean = 0.06%) than those from the nonendemic area (0.12%) (P < 0.05). However, at any parasite density, both clinical disease and pathology were significantly less in the endemic area patients (P < 0.001, for both clinical score and ESR), indicating that the clinical immunity seen in the endemic area patients was a true tolerance of parasites. Although plasma TNF-α levels were elevated in both groups of patients, they were significantly higher in the nonendemic area patients than in patients from the endemic area (P < 0.01). Furthermore, at comparable levels of plasma TNF-α, nonendemic area patients had both a higher intensity of clinical disease and an underlying pathology than those from the endemic area, suggesting that if TNF-α is indeed a mediator of clinical disease, the endemic area patients may be tolerant to its effects. Hypoglycemia was not observed in any of these P. vivax patients despite some with high levels of plasma TNF-α.

In regions where malaria is highly endemic, as in most areas of tropical Africa, it is young children who suffer high morbidity and mortality due to this disease. In older children and adults, in spite of experiencing continued high malaria inoculation rates, most individuals, with the frequent exception of pregnant women, rarely experience symptoms. It can be said of these older age groups that a high degree of clinical immunity to malaria infection has been achieved. The nature of this clinical immunity appears to have two distinct components. The first to be acquired is suppression of the symptoms of disease in spite of the continuing presence of malaria parasites in the blood, often at high levels. This is the condition of clinical tolerance of malarial infection that has been often noted in the older African child and has not been based on quantitative measurement and analysis. The second form of clinical immunity is only reached by late childhood or adolescence after further years of such exposure. In this condition, a high degree of anti-parasitic immunity is achieved such that parasite densities in the blood are suppressed to very low often subdetectable levels. In areas of low malaria endemicity, such as in Sri Lanka and most malarious areas of the Indian subcontinent, full, exposure-acquired anti-parasitic immunity to malaria is rarely if ever achieved. It is generally known, however, that the intensity of the common symptoms of malarial infections are less pronounced among residents of malaria-endemic areas of Sri Lanka than in individuals from nonendemic areas who became infected following brief visits to an endemic locality. This suggested that a degree of clinical immunity to malaria might exist among residents of malarious localities in Sri Lanka. In the present study, we have investigated this possibility. Our approach has been to measure clinical disease and the parasitologic and hematologic status of malaria patients presenting to clinics in endemic and nonendemic areas of Sri Lanka. To do this we devised a system to quantify the intensity of disease based on patient interview. These data, together with parasitologic and selected hematologic measurements, have been analyzed to determine the extent and nature of the differences in clinical malaria in the patients from endemic and nonendemic localities. While most previous evidence for the existence of clinical immunity to malaria has been derived from comparison of clinical disease and parasitemia in different age groups in the same population, this study is based on two age-matched populations who have had very different degrees of exposure to malaria.

PATIENTS, MATERIALS, AND METHODS

Patients and controls. Patients with Plasmodium vivax infections were studied from two localities. Colombo. Forty-three adult patients 13–69 years of age (median age = 26 years) attending the General Hospital in Colombo. All were residents of Colombo, which is located in a region of Sri Lanka where malaria transmission does not occur. These nonendemic area patients had acquired their infections following travel to a malaria-endemic region of the country. In most individuals, this was their first malaria infection although a few had experienced a single previous malaria infection at some time prior to their present attack. The P. vivax parasitemias ranged from 0.006% to 0.43% at
the time of first attendance at the General Hospital. As controls for the nonendemic area patients, 43 age-matched, malaria-uninfected individuals who were resident in Colombo and were not experiencing any illness at the time were chosen.

Kataragama. Thirty-nine cases of \textit{P. vivax} malaria, age-matched to the nonendemic area patients in this study, were from among residents of a malaria-endemic locality at Kataragama in southern Sri Lanka. The ages of these patients ranged from 13 to 67 years (median age = 23.5 years). By their own accounts, the patients in this group had experienced several previous malarial infections (range = 3–50, median = 8); these could have been \textit{P. vivax}, or to a lesser extent, \textit{P. falciparum}, with both parasite species having been prevalent, and with \textit{P. vivax} having higher inoculation rates in the Kataragama area during the period of this study. The parasitemias of their current \textit{P. vivax} infections ranged from 0.004% to 0.3%. As controls for the endemic area patients, 43 age-matched, malaria-uninfected individuals who were resident in Kataragama and were not experiencing any illness at the time were chosen.

Informed voluntary consent was obtained from all patients and controls before being included in this study. Ethical clearance for this study was granted by the Ethical Committee of the Faculty of Medicine of the University of Colombo (Colombo, Sri Lanka).

The two study areas, Colombo and Kataragama, are situated 450 km from each other; there are no significant differences in the prevalence of infectious diseases other than malaria between these two areas. Patients from both the endemic and nonendemic area groups belonged to the same range of social and economic classes; this is further supported by the fact that patients in both groups were those who sought treatment at Government health care institutions, which provide health services free of charge. Patients from both areas were self-selected in the sense that they had presented themselves to a clinic for diagnosis.

In Colombo, most of the patients presented themselves directly to the Outpatients Department of the Colombo General Hospital with symptoms of a febrile-type illness and a history of recent travel outside the Colombo region. All had thick and thin blood films made and stained with Giemsa and a diagnosis of malaria was made following microscopic examination. The 43 \textit{P. vivax} patients from Colombo were consecutively diagnosed in this way and included in the study without further selection.

The patients from Kataragama also presented themselves directly to a field clinic in the area with symptoms of a febrile-type illness. All were examined by microscopy for malarial infection on thick and thin blood films stained with Giemsa and a diagnosis of malaria was made following microscopic examination. The 39 \textit{P. vivax} patients from Kataragama who were included in the study were chosen to be as nearly as possible age-matched to the Colombo patients. Within this constraint, the Kataragama patients were included into the study consecutively, i.e., in the order in that they were diagnosed.

Clinical assessment. The spectrum and severity of clinical disease in the malaria patients was assessed quantitatively using a questionnaire (form CL1.90) developed for the purpose. The questionnaire addressed 11 symptoms that commonly accompany a malarial infection, namely, headache, myalgia, arthralgia, shivering, cold, sweating and hot spells, nausea, vomiting, anorexia, backache, and hypochondriac pain. A patient’s perception of the degree of severity of the first four of these symptoms, headache, myalgia, arthralgia, and shivering, was evaluated as being either absent, mild, moderate, or severe; the remaining symptoms were evaluated as absent, mild, or severe. In recording the patient’s response for each symptom, a numerical score of 0, 1, 2, or 3 was assigned according to whether the symptom was perceived as absent, mild, moderate, or severe, respectively. The sum of the scores of all 11 symptoms was taken as the total clinical score of a patient. This system enabled numerical comparisons and analyses to be conducted on the clinical states of the patients.

The patients were also asked to indicate the degree of absence from work associated with their present illness as either total, partial, or none. To standardize against subjective bias in patients’ responses due to differences in tolerance of symptoms and thresholds for pain, each patient was asked to assess the general severity of their present illness in relation to previously experienced febrile illnesses other than malaria, such as viral fevers, with the response being mild, moderate, or severe.

In developing the patient questionnaire, the list of clinical symptoms was decided upon after a review of the literature on clinical manifestations of acute malarial infection (excluding severe or complicated cases). Those chosen for inclusion were the 11 symptoms most commonly identified as being associated with uncomplicated malarial infection. Pilot studies were conducted to validate the use of the questionnaire, which was administered by three trained investigators using Sinhala, the mother tongue of the patients in both localities in this study. During the pilot studies, the interview method of the investigators was tested to ensure standardization of the patients’ responses among the investigators.

Physiologic and hematologic measurements. The oral temperature of each patient was recorded at the time of questioning using a mercury clinical thermometer.

Venous blood samples (6 ml) were collected from each patient and control for measurement of the erythrocyte sedimentation rate (ESR) using the standard Westergren method. The blood glucose levels and serum bilirubin levels were measured using an enzymatic assay and a chemical assay, respectively (Gilford Diagnostic Methods, Corning, OH). The reticulocyte counts were made using standard methods.

Plasma tumor necrosis factor-α (TNF-α) measurements were made using 1 ml of blood per subject that was added to 10 μl of 10% sodium EDTA and 20 μl of the protease inhibitor aprotinin (0.6 trypsin inhibitor units/ml; Sigma, St. Louis, MO). The samples were processed immediately at room temperature by centrifugation at 12,000 × g for 10 min. Plasma was separated and stored at −20°C until further use. The TNF-α measurements were made using the immunoradiometric assay previously described. Kits used in this assay were kindly provided by Medgenix (Fleurus, Belgium).

Statistical analysis. Statistical comparisons between groups of patients and/or controls were made using the Mann-Whitney U test. Partial correlation coefficients were
obtained after controlling for confounding factors, e.g., parasitemia using multiple linear regression analysis. Correlation coefficients (r values) were calculated using the Spearman rank correlation.

RESULTS

Severity of disease as determined by clinical interview of endemic and nonendemic area malaria patients. The clinical presentation of *P. vivax* patients from an endemic area (Kataragama) and a nonendemic area (Colombo) of Sri Lanka has been studied. All 11 symptoms identified in a clinical questionnaire were experienced by individuals within both groups of patients (Figure 1). For most symptoms, the prevalence and the intensity were markedly lower in the group from the endemic area than in those from the nonendemic area. Total clinical scores of the endemic area patients ranged from 0 to 22 (mean = 8.8), and those from nonendemic area patients ranged from 5 to 31 (mean = 19) (*P < 0.01*). Severity scores for nausea and vomiting were those most reduced in the endemic area patients (by 78%); there was a similar relative reduction in severity of myalgia (by 74%). Less reduced were the other symptoms associated with a paroxysm, i.e., chills, rigors and sweating (by 57%), body pains, i.e., hypochondrial pain, backache, and arthralgia (by 56%), and headache (by 38%). Anorexia showed little difference in severity in the two groups of malaria patients, being quite pronounced in both. The extent of absence from work reported to have resulted from their current malarial illness reflected the perceived degree of clinical severity in the patients from the endemic and nonendemic areas, respectively (Table 1).

We considered the possibility that the levels of clinical severity reported at the interview and their extent of absence from work might reflect attitudes that differed between the patients from endemic and nonendemic areas. To control for this, we asked all patients how the severity of their current malarial infection compared with that of nonmalarial febrile illnesses that they had previously experienced. These illnesses would have included influenza and other viral infections of which the two groups would have had similar previous experiences. Patients from the endemic area, whose average malaria clinical scores were low, indicated that their malaria symptoms were generally mild compared with other febrile illnesses (Table 2). Patients from the nonendemic area, whose average malaria clinical scores were high, indicated that their malaria symptoms were generally severe in comparison with other illnesses. Overall, the clinical score of patients correlated positively with the score of this question (r = 0.54, *P < 0.01*). This indicates that differences in clinical score between the endemic and nonendemic area groups are not due to a general bias in perceptions of the severity of disease symptoms between the groups.

The average densities of blood-stage malaria parasites (% parasitemias) in the endemic area patients (mean = 0.06%) were significantly lower than in the nonendemic area patients (mean = 0.12%) (*P < 0.05*). However, these differences in parasite density were clearly not the basis for the difference in clinical score between the two groups. The parasitemias

<table>
<thead>
<tr>
<th>Question</th>
<th>Not at all</th>
<th>Partial</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>What was the degree of absence from work during the present illness?</td>
<td>15.7</td>
<td>80.4</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>34.1</td>
<td>63.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Endemic area</th>
<th>Nonendemic area</th>
</tr>
</thead>
<tbody>
<tr>
<td>How does the severity of symptoms of the present illness compare to that of previous febrile nonmalarial illnesses?</td>
<td>74.5</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>7.8</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>22.7</td>
<td>72.7</td>
</tr>
</tbody>
</table>
covered a wide range in both groups (Figure 2) and even after controlling for differences in parasite density, the clinical scores of the endemic area patients were significantly lower than those of the nonendemic area patients. A multiple linear regression analysis indicated that at any given parasite density, the clinical scores of the endemic area patients tended to be 10 points lower than those of the nonendemic area patients ($P < 0.001$; regression coefficient $r = 10.01$).

**Pathophysiologic measurements.** Measurement of the ESR, serum bilirubin levels and reticulocyte counts were used as indicators of underlying pathologic changes; TNF-$\alpha$ and blood glucose levels were also measured in all malaria patients and uninfected control individuals in the study. In the nonendemic area patients, the ESR values were markedly and significantly elevated compared with either their own controls ($P < 0.001$) or those of the endemic area patients ($P < 0.001$). The ESR values in the endemic area patients were, however, not significantly different when compared with the values of their uninfected (endemic area) controls ($P = 0.05$ and $P = 0.06$, respectively). Although there was no significant direct correlation between the TNF-$\alpha$ levels and the clinical score in either group of patients, among the nonendemic area patients, those who had TNF-$\alpha$ levels greater than 250 pg/ml ($n = 11$) had a significantly higher clinical scores (mean = 29) than those who had lower TNF-$\alpha$ levels ($n = 32$, mean = 19.3) ($P = 0.02$). In endemic area patients, in whom the TNF levels were generally very low, no such correlation was found. A notable finding was that when the TNF-$\alpha$ levels of all patients were examined in relation to the intensity of clinical disease (clinical score) or the underlying pathology (ESR), at any given plasma TNF-$\alpha$ level, the nonendemic area patients had a higher clinical score (Figure 2) and a higher ESR than those from the endemic area (multiple linear regression, $P < 0.001$, $r = 9.98$ for clinical score, and $P < 0.001$ and $r = 26.89$ for ESR).

Blood glucose levels were within the normal range in all groups. In the endemic area uninfected controls, the blood glucose levels were slightly but significantly lower (Table 3). The randomly measured body temperature, which was measured at the time of patient interview rather than at the peak of a paroxysm, was marginally lower in endemic area

![Figure 2. Relationship between the intensity of disease (clinical score) and parasite density in blood (% parasitemia) in endemic (●) and nonendemic (○) area patients.](image)

**Table 3**

Results of laboratory investigations on blood from acute *Plasmodium vivax* malaria patients from endemic and nonendemic areas and their respective age-matched, healthy controls

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Median (range)</th>
<th>Median (range)</th>
<th>Significance of the difference between the two groups of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonendemic area</td>
<td>Endemic area</td>
<td></td>
</tr>
<tr>
<td>ESR, 1st hour (mm)</td>
<td>5 (2–55)</td>
<td>6 (2–72)</td>
<td>0.27 &lt;0.001</td>
</tr>
<tr>
<td>Serum bilirubin (mg/100 ml)</td>
<td>0.7 (0.2–1.1)</td>
<td>0.6 (0.3–1.8)</td>
<td>&lt;0.001 0.02</td>
</tr>
<tr>
<td>Reticulocyte count (/µl)</td>
<td>48,465 (32,550–70,560)</td>
<td>46,000 (33,320–93,000)</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma TNF (pp/ml)</td>
<td>27 (15–270)</td>
<td>ND 60</td>
<td>0.009</td>
</tr>
<tr>
<td>Blood glucose (g/100 ml)</td>
<td>96.3 (64–150.8)</td>
<td>78.2 (64.2–160.6)</td>
<td>0.006 0.7</td>
</tr>
</tbody>
</table>

* ESR = erythrocyte sedimentation rate; TNF = tumor necrosis factor; ND = not done; NO = not obtained.

† By Mann-Whitney U test. P1 and P2 = significance of the difference between the controls and patients in nonendemic and endemic groups, respectively.
patients than in those from the nonendemic area (median = 37.3°C and 38.3°C for endemic and non-endemic area patients, respectively), although a level of statistical significance was not attained for this difference.

**DISCUSSION**

We have conducted a study of the clinical presentations of acute *P. vivax* malarial infection in patients from malaria endemic (Kataragama) and nonendemic (Colombo) regions of Sri Lanka. The study involved assessment of the severity of 11 symptoms recognized in the literature as being associated with acute uncomplicated malarial infections. Assessment was made by patient interview, and the responses for each symptom (absent, mild, moderate, or severe) were recorded on a severity scale of 0 to 3. The interview technique was standardized to achieve comparable results between the two groups of patients. An internal control for different perceptions of severity of illness was included by recording each patient’s perception of the severity of their current malarial infection in comparison with that of previous nonmalarial febrile illnesses such as viral fevers.

The symptom severity scores showed that the overall severity of their infections as perceived by the nonendemic area patients (mean total clinical score = 19) was much greater than those in the endemic area patients (mean total clinical score = 8.3). The validity of this difference was supported by the finding that in comparison with previously experienced nonmalarial febrile illnesses, patients in the nonendemic area group generally indicated that their current infection was severe while in endemic area group it was generally believed to be mild. Since their experiences with other febrile illnesses, such as influenza and viral infections, would have been similar in the two populations, this finding suggests that the intensities of symptoms associated with their malarial infections were genuinely different between the two populations. Our finding that the ESR of patients, which is a general measure of underlying pathology, correlated strongly with the clinical score, which further substantiates the scoring system as a valid measure of the intensity of clinical disease. Therefore, we conclude that the patients from the endemic area population had a tolerance of *P. vivax* malarial infection relative to the patients from the nonendemic area population.

This reduced intensity of symptoms in the endemic area patients could have been due to acquired immunity against the blood-stage parasites, leading to lowered densities of the parasites in the blood. However, this cannot be the explanation. Although the mean parasite densities in the nonendemic area patients were about twice those of the endemic area patients, there was a highly significant reduction in the clinical score at any parasite density in the endemic area patients compared with the nonendemic area patients. Thus, the relative clinical tolerance of infection experienced by the endemic area patients was a true tolerance of the presence of parasites and not simply due to the reduction in parasite densities.

In addition to the clinical assessment of patients by interview, we made hematologic measurements that indicate the degree of underlying pathology in malaria, i.e., of the ESR and serum bilirubin levels, which reflect the degree of hemolysis, and reticulocyte counts, which are generally elevated in response to the anemia caused during malaria infections. The ESRs were greatly elevated in the nonendemic area patients but were within the normal range in the endemic area patients. Both serum bilirubin levels and reticulocyte counts, which were elevated in malaria patients compared with their respective controls, were affected to a significantly lesser degree in endemic area patients than in those from the nonendemic area; these differences were maintained even when the differences in the parasitemias between the two groups were controlled for. These results confirm that in addition to the clinical tolerance of parasites in the endemic area group of patients, there was also a lower degree of underlying pathology in them at comparable levels of parasitemia.

Plasma TNF-α and blood glucose levels were also measured in the patients and controls. In contrast to the ESR, plasma TNF-α levels were elevated in malaria patients in both groups but were significantly higher in those of the nonendemic area than in the endemic area group. We further analyzed the relationship between TNF-α levels in patients’ plasma and the severity of disease; although there was no significant and direct correlation between plasma TNF-α levels and the clinical score in either group, in the nonendemic area patients, in whom TNF-α levels spanned a wide range, those who had very high levels of TNF-α also had significantly greater intensities of clinical disease, and to this extent, the intensity of clinical disease in these patients correlated with their plasma TNF-α levels. This evidence is consistent with previous findings, which suggest that TNF-α is a disease-mediating cytokine in malaria. 7,11 We have persuasive evidence from previous studies of the causative role of TNF-α in the paroxysm of malaria.16 In this case, the period of a paroxysm was associated with the sharp increase and subsequent decrease in TNF-α levels; however, the absolute level of plasma TNF-α reached varied widely among paroxysms of similar intensity between patients; it is therefore not surprising that the absolute levels of TNF-α in plasma did not correlate with the disease intensity of individual patients to a greater extent than we found here.

An important finding was that at any particular level of plasma TNF-α, both the intensity of clinical disease as well
as the underlying pathology were higher in the nonendemic area patients than in the endemic area patients; this implies that if TNF-α is indeed involved in the pathogenesis of uncomplicated malaria, the endemic area patients had acquired a tolerance to TNF. Tolerance to TNF has been demonstrated in animals, and has been suggested to occur in humans based on the evidence that when tumor patients were treated with TNF-α, they became refractory to its side effects after repeated exposure. Therefore, the evidence presented in this study, may be the first to support the existence of tolerance to autologous TNF-α in humans. Tolerance to TNF is likely to be one of several mechanisms underlying the acquisition of clinical immunity. We have previously demonstrated two other mechanisms that operate in the clinically immune and which may therefore contribute to a reduction in the intensity of disease, they being, 1) a serum-mediated neutralization of parasite exoantigens that are released at schizont rupture and that induce cytokine production, and 2) a down-regulation of mononuclear cell function reducing their ability to produce cytokines in response to stimulation with parasite exoantigens (Karunaweera ND, 1993, PhD thesis, University of Colombo, Sri Lanka).

Although this particular study focused entirely on uncomplicated P. vivax malaria, high levels of TNF-α have also been reported to correlate with the severity of complicated infections of P. falciparum. It is noteworthy that in P. vivax infections, none of which had severe or complicated features, we have found similar, and, during paroxysms, much higher, blood TNF-α levels than those reported elsewhere in association with P. falciparum cerebral malaria.

Hypoglycemia has been commonly reported in association with P. falciparum infections. In the present study, hypoglycemia was notable by its absence in any of the P. vivax patients we studied, as indicated by the normal blood glucose levels during infection. It has been suggested that TNF-α, whose levels are usually elevated during acute malarial infections, is a likely mediator of this effect. In this study, however, it is clear that there was no trend towards lowered blood glucose levels in either the endemic or the nonendemic area patients despite plasma TNF-α levels being high. Also, there was no correlation between levels of TNF-α and blood glucose levels in either group (r² = 0.0006, P = 0.8).

We have introduced a quantitative method to assess the intensity of clinical disease in uncomplicated malaria, and have substantiated its validity by several methods, including the use of pathologic measurements. This method of scoring the intensity of clinical disease has since been used in larger samples of patients in several investigations on the pathogenesis of malaria, enabling new insights to be made to this area of study (Gunawardena DM and others and Pathirana SL and others, unpublished data). Using the clinical score, we have demonstrated that an immunity against clinical disease, by way of a true tolerance of parasites, as manifested by a lowered intensity of clinical symptoms as well as by a reduced degree of pathology, exists in the population in the malaria-endemic area of Kataragama. Whether clinical immunity is age-acquired, i.e., characterized by a cumulative immunologic memory (whether it is sustained only by frequent malaria blood infections) was not revealed by this investigation. It was striking that within the endemic area group of patients, no obvious evidence could be found for an increasing clinical immunity with age. More extensive longitudinal studies are underway in this endemic area population to investigate the nature of this immune response, which underlies the acquisition of clinical immunity to malaria.

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