Brief Definitive Report

Transient Increase in Circulating γ/δ T Cells during *Plasmodium vivax* Malarial Paroxysms

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Summary

The percentage of peripheral blood mononuclear cells (PBMC) bearing the $CD3^+$ phenotype and the α/β and γ/δ T cell receptors (TCR) in PBMC were examined in *Plasmodium vivax* malaria patients and convalescents. The cells were labeled with monoclonal antibodies, stained with either fluorescene or phycoerythrin, and examined by ultraviolet (UV) microscopy. A highly significant increase in both the proportion and the absolute numbers of γ/δ T cells ($p < 0.005$ and < 0.001, respectively, Student's t test) was observed in nonimmune *P. vivax* patients during clinical paroxysms compared to nonmalarial controls. These T cells, which normally constitute not more than 3–5% of PBMC, constituted $\leq 30\%$ of PBMC during paroxysms in these nonimmune patients in whom the clinical symptoms were severe. A less significant increase of γ/δ T cells were also observed in these nonimmune patients during infection, between paroxysms and during convalescence. In contrast, in an age-matched group of semi-immune patients resident in a malaria-endemic region of the country, in whom the clinical disease was comparatively mild, there was no increase in γ/δ T cells either during infection, even during paroxysms, or convalescence. The severity of disease symptoms in patients as measured by a clinical score correlated positively with the proportion of γ/δ T cells in peripheral blood ($r = 0.53$, $p < 0.01$), the most significant correlation being found between the prevalence and severity of gastrointestinal symptoms, nausea, anorexia, and vomiting, and the proportion of γ/δ T cells ($r = 0.49$, $p = 0.002$). These findings suggest that γ/δ T cells have a role to play in the pathogenesis of malaria, possibly in the general constitutional disturbances and particularly in gastrointestinal pathology in malaria.

T lymphocytes, which express the CD3 marker and lack CD4 and CD8 markers, have been shown to lack mature messenger (m)RNA for α and β genes and express the γ and δ genes and proteins (1). Such lymphocytes expressing the TCR-γ/δ constitute a minor subpopulation in the peripheral blood of adults, and many of them reside in the spleen, and to a lesser extent in the thymus, tonsils (2), and the epithelia of the large intestine (3) and lung (4). The biological role of the cells bearing the TCR-γ/δ is as yet unclear, but there are indications that some γ/δ T cells can mediate cytotoxicity (4). γ/δ T cells behave like α/β T cells in that they secrete the lymphokines IL-2, IFN-γ, TNF-α and β, and express cytotoxic activity (5–7).

Recent evidence has led to speculation about an involvement of γ/δ T cells in immunity and/or immunopathology in human malaria. The frequency of γ/δ T cells in the peripheral blood of acute *Plasmodium falciparum* malaria-infected individuals has been shown to increase, and remain elevated for several weeks during convalescence (8). In nonimmune individuals, a vast majority of T cells vigorously proliferating in response to freeze-thawed extracts of *P. falciparum* blood stage parasites in vitro have been shown to be γ/δ T cells (9–11), and are the source of a significant fraction of TNF-α and IFN-γ that were produced in these cultures (12).

There is much recent evidence to suggest that TNF and other cytokines are involved in the clinicopathological effects of malaria (13–17). We have shown that individuals living in malaria-endemic regions acquire a clinical (antidisease) immunity to malaria which is distinct from antiparasite immunity. In acute *Plasmodium vivax* malaria, patients among residents of malaria-endemic regions in Sri Lanka, both the intensity of the clinical disease and the underlying pathology were considerably less than in nonimmune individuals experiencing a primary infection (Karunaweera, N. D., R. Carter, G. E. Grau, and K. N. Mendis, manuscript submitted for publication). In this study we examined the lymphocyte composition in the peripheral blood of acute *P. vivax* patients who were either clinically non- or semi-immune, and who reside in a malaria-nonendemic and -endemic region, respectively, paying particular attention to paroxysms, the prominent peri-

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odic clinical events that occur during the course of an acute malaria infection. We report here that profound changes occur in the composition of PBLs during a malaria infection in the nonimmune patient, particularly with respect to γ/δ T cells which normally constitute an insignificant component of PBLs.

Materials and Methods

Patients. PBMC were studied in adult *P. vivax* patients (*a*) during the course of an acute infection soon after diagnosis and before antimalarials were administered; and (*b*) during convalescence 4–6 wk after a *P. vivax* infection was drug treated. Patients and convalescents were studied from two groups viz: (*a*) residents of the malaria-nonendemic area of Sri Lanka (Colombo and its suburbs) (18) who had acquired the infection on a visit to an endemic region, many of whom had not experienced a malaria infection previously (see Table 1); and (*b*) residents of Kataragama, a *P. vivax* malaria-endemic region in southern Sri Lanka (19), who had experienced many previous infections of malaria. In both groups of acute *P. vivax* patients, cells were examined either at or between paroxysms. Paroxysms are periodic clinical events that coincide with schizont rupture and that are characterized by a sharp rise and fall of body temperature accompanied by chills, rigors, and sweating, in that order (16). In most endemic patients, the paroxysms were not as clinically pronounced as in the nonendemic patients, and in both groups of patients, the time of schizont rupture as monitored on a Giemsa-stained blood film served as a guide to the onset of a paroxysm. An age-matched group of healthy individual Colombo residents who had no past history of malaria, were studied as controls.

In all subjects, a malaria diagnosis was made by microscopic examination of a blood film. A complete history was taken, and in the case of patients (but not convalescents) the clinical state was evaluated by the use of a questionnaire (Clinical Evaluation Form CL1.90) which has been developed and validated by us and described previously (Karunaweera, N. D., R. Carter, G. E. Grau, and K. N. Mendis, manuscript submitted for publication). Briefly, the prevalence and degree of severity of each of 11 symptoms (headache, myalgia, arthralgia, shivering, cold, sweating, nausea, vomiting, anorexia, backache, and hypochondrial pain) were determined as perceived by the patient and assigned a score ranging from 0 to 3 if it was absent, mild, moderate, or severe, respectively. Thereafter, a total clinical score was derived for every patient, this being the sum of the scores of the individual symptoms. The clinical assessment was introduced when the study was in progress, therefore clinical scores are available only for a proportion of patients.

Preparation, Staining, and Analysis of PBMC. After voluntary informed consent, patients were bled by venepuncture into heparin (10 U/ml) and PBMC were isolated using Lymphoprep™ (Nycomed AS, Oslo, Norway) as previously described (20). Total mononuclear cell counts were made in a hemocytometer and washed cells were resuspended in PBS, with 0.1% sodium azide and 2% FCS at a concentration of 2×10^7 cells/ml; aliquots containing 10^6 cells, each stained with mAbs directed against Leu-4 (anti-CD3), Leu-3 (anti-CD4), and Leu-2 (anti-CD8), all fluorescein conjugated; and TCR-γ/δ, which was PE conjugated (Becton Dickinson & Co., Mountain View, CA) using standard methodology (11). Aliquots of stained cells were each resuspended in 20 μl of mounting medium (PBS containing 0.1% sodium azide and 50% glycerol) and 10 μl was placed under a 22 × 22-mm coverslip and examined at a magnification of 400 on a Leitz Ortholux fluorescence microscope. A minimum of 360 stained cells or 15 microscope fields (whichever was lower) was examined. Analysis of stained cells from five patients using a FACS® (Becton Dickinson & Co.), confirmed that results of direct microscopy by eye was equally reliable.

Results

Individuals in the three study groups were comparable in age, but differed markedly with respect to their past malaria experience (Table 1). Patients and convalescents from Kata-

Table 1. *Description of Test and Control Groups**

	Control‡	Nonendemic	Endemic	*p* value§
Number of subjects	8	25	42	
Age range in years (median)	22–42 (37)	16–65 (28)	14–56 (28)	NS
Previous malaria infections				
Range (median)	0	0–1 (0)	2–20 (10)	<0.001
Percent parasitaemia‖				
Range	NA	0.004–0.57	0.001–0.34	<0.01
Mean (± SD)		0.14 (±0.14)	0.044 (± 0.073)	
Clinical score¶				
Score range (mean)	NA	14–33 (20.88)	6–25 (13.5)	<0.005

* Test groups include patients (during and between paroxysm) and convalescents.
‡ Nonmalarial controls.
§ Significance of difference between endemic and nonendemic subjects (Students *t* test) (during infection and paroxysm) is given here.
‖ Parasitaemias of patients only, excluding convalescents, are given here (n = 18 in nonendemics; n = 24 in endemics). Mean = arithmatic mean.
¶ The clinical scores (given in points) were available only in 13 nonendemic and 33 endemic patients. Mean = geometric mean.
NA, not applicable; *NS* ($p > 0.05$).

ragama, the endemic region, had experienced many more past malaria infections, had significantly lower parasite densities in blood, and experienced less intense clinical symptoms as indicated by their lower clinical score than did nonendemic patients, a majority of whom had no previous experience of malaria at all (Table 1). Endemic patients thus showed evidence of both an antiparasite and a clinical or antidisease immunity.

An analysis of the cell types among PBMC revealed a significant increase in the proportion of γ/δ T cells in nonimmune patients during clinical paroxysms. These cells, which normally represent not more than 3–5% of PBMC, constituted up to 26% of the circulating mononuclear cell population during a paroxysm in nonimmune patients (Fig. 1). A moderate but less significant increase in the proportion of γ/δ T cells was also observed during infection (between paroxysmal events) and convalescence in these nonimmune patients compared to nonmalarial controls (Fig. 1). In none of the endemic patients, however, was there a significant elevation of the proportion of γ/δ T cells beyond the levels which prevailed in nonmalarial controls either during infection (at or between paroxysms) or convalescence. Thus, the proportion of γ/δ T cells in the nonimmune patients during paroxysms was significantly higher than that of the endemics at a corresponding time of the infection ($p < 0.005$, Student's t test) or of any other category of individuals studied, including nonmalarial controls.

The absolute γ/δ cell counts of the nonimmune patients were also significantly elevated during a paroxysm compared to those of normal controls ($p < 0.001$). This was so, despite a significant reduction in the total mononuclear cell counts which occurred in all patients, particularly during a paroxysm (Table 2). The difference in the absolute γ/δ cell counts between nonendemic and endemic patients at the time of paroxysms was thus as significant as the difference between their proportions ($p < 0.005$; Table 2).

In the patients (both endemic and nonendemic) in whom the clinical scores were available, the total clinical scores (a measure of disease severity) correlated positively with the percent proportion of γ/δ T cells in peripheral blood ($r = 0.53$, Spearman Rank Correlation, $p < 0.01$; Fig. 2). When individual symptoms were analyzed, the highest correlation with the proportion of γ/δ cells was obtained with symptoms relating to the gastrointestinal tract, namely, anorexia, nausea, and vomiting, each of which gave a significant correlation on their own with γ/δ T cells, as well as when they were combined ($r = 0.49$ and $p = 0.002$), the best correlation having been obtained with the prevalence of vomiting ($p = 0.007$). Even when these three symptoms were removed from

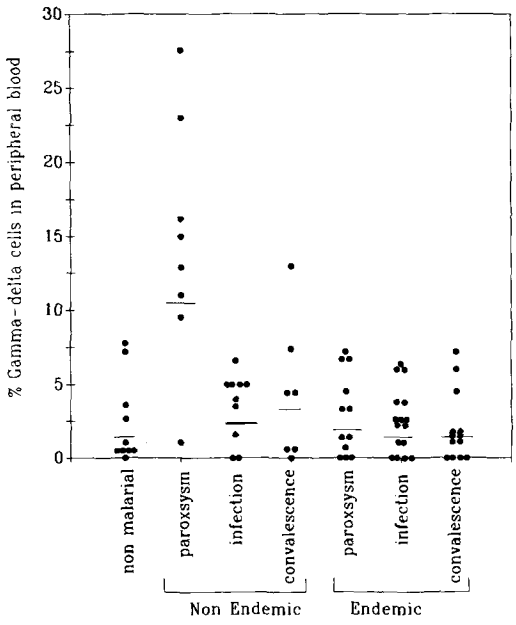

Figure 1. The percentage of γ/δ T cells (●) among PBMC in acute malaria patients from a malaria-nonendemic, and an endemic region during and between paroxysms, in comparison with those in nonmalarial controls from the nonendemic region. (——) Arithmetic mean.

Table 2. *Absolute Cell Counts of Test and Control Groups**

	Geometric mean ($x/\div$ SD) of cell counts/μl in						
		Nonendemic			Endemic		
Cell type	Controls	Paroxysm	Infection	Convalescence	Paroxysm	Infection	Convalescence
Mononuclear	1,210 ($x/\div 0.21$)	1,053 ($x/\div 0.23$)	1,237 ($x/\div 0.13$)	1,601 ($x/\div 0.15$)	806 ($x/\div 0.14$)	946 ($x/\div 0.20$)	1,796 ($x/\div 0.15$)
γ/δ	18.33 ($x/\div 0.52$)	110.3 ($x/\div 0.28$)	46.3 ($x/\div 0.36$)	61.26 ($x/\div 0.43$)	10.57 ($x/\div 0.67$)	9.07 ($x/\div 0.7$)	42.29 ($x/\div 0.78$)

The difference between the absolute total mononuclear cell counts of nonendemic patients (during infection and paroxysms) and endemic patients was significant ($p < 0.05$).
The difference between the absolute γ/δ T cell counts of nonendemic and endemic patients at paroxysm was highly significant ($p < 0.005$).
* Test groups include patients (during and between paroxysm) and convalescence.

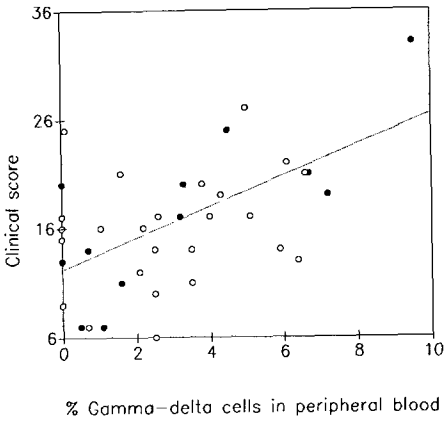

Figure 2. The relationship between the severity of clinical disease (*clinical score*) and the percentage of γ/δ T cells among PBMC during infection in patients from the malaria-nonendemic and -endemic regions. In some patients, the cell counts were made during a paroxysm, $n = 12$ (●), and in others during infection between paroxysms, $n = 25$ (○). The single regression line applies to all values; $r = 0.53$ and $p = <0.01$ (Spearman Rank Correlation).

the total score, the correlation with the proportion of γ/δ T cells was still evident, though less significant ($r = 0.377$ and $p = 0.023$).

The differences in the absolute γ/δ T cell counts and clinical scores of patients between the non- and semi-immunes were not due to differences in the parasite density between the two groups. Multiple linear regression analyses in which the degree of parasitaemia was controlled, revealed that at any given parasite density, the γ/δ cell count would be 74 cells/μl higher ($p = 0.0025$), and the clinical score six points higher ($p = 0.0072$) in the nonendemic than in the endemic patients.

Double staining of cells confirmed that cells which stained with γ/δ markers were CD3 positive and negative with α/β markers. Consequently, nonimmune patients at paroxysm in whom the greatest increase in γ/δ T cells occurred, recorded the lowest proportion of α/β T cells (data not shown).

Discussion

A significant increase in both the proportion and absolute numbers of γ/δ T cells was observed in the circulation of nonimmune patients during a paroxysm. This increase appears to be transient, because at no other stage of infection or convalescence were these cells detected in such large numbers. Equally striking was the complete absence of such an increase in the immune patient even during a paroxysm. A previous study (8) demonstrated an increase in the number and proportion of γ/δ T cells during the course of *P. falciparum* infections, though the data was not given in relation to the stage of the parasite's development in the erythrocytic cycle as presented here. We demonstrate here that in *P. vivax* infections, both the proportion and the absolute numbers of γ/δ T cells increase, and that this increase is associated with the time of schizont rupture and the event of a paroxysm. We have previously described several other transient events in the circulation of nonimmune patients during the event of a paroxysm which are evoked by the rupture of schizont-infected erythrocytes. One, was a sharp but transient rise, and then fall of circulating plasma TNF-α levels in close parallel to the rise and fall of body temperature that occurs during a paroxysm (16). The other, was the detection of a transient parasite-inactivating activity in the plasma during the peak of a paroxysm. This activity was dependent on both plasma TNF and an as yet unidentified plasma factor(s), the presence in plasma of which is very transient (15). The malarial paroxysm is therefore an event marked by several pronounced and transient changes in the circulation of the nonimmune patients. In the immune patients, not only is the intensity of the paroxysms less, but the accompanying changes, including the rise in plasma TNF, the parasite-inactivating effects, and as reported here, the increase in circulating γ/δ T cells, are much less apparent (15).

At least two independent observations made in this study suggest that γ/δ T cells could play a role in the pathogenesis of malaria. The first, is the pronounced and transient increase in γ/δ T cells in the circulation of clinically nonimmune patients during paroxysms, and the absence of this phenomenon in the clinically immune patients in whom the disease runs a milder course and in whom paroxysms are distinctly less intense. The second is the significant correlation between the proportion of γ/δ T cells in peripheral blood and the intensity of clinical disease in patients in general. The incidence of γ/δ cells in the peripheral circulation correlated best with the prevalence and intensity of gastrointestinal symptoms, which viewed in the light of their normal residence in intestinal epithelium suggest that their activity may be linked to intestinal pathology in malaria. The transient increase in γ/δ T cells in the circulation is likely to be the result of recruitment of cells into the circulation from tissue sources in response to parasite exo-antigens which are released during schizont rupture in vivo. These cells may be a source of the cytokines that are transiently and markedly elevated during a paroxysm in the nonimmune patient. Alternatively, cytokines produced during a paroxysm might themselves provide the stimulus for the recruitment of γ/δ T cells from their sequestered sites into the circulation.

These findings support the view that γ/δ T cells may be involved in the pathogenesis of malaria (12), at least in the constitutional disturbances such as paroxysms and possibly in symptoms that relate to gastrointestinal disturbances that accompany the disease in acute uncomplicated malaria.

Endemic patients in whom the disease was much less intense were characterized not only by the absence of an increase in γ/δ T cells, but also by a general lymphopenia which was more pronounced than in the nonendemic patients. We have previously suggested that one of the mechanisms of acquiring clinical immunity was by downregulating cells involved in pathogenesis (20, Karunaweera, N. D., P. Gamage, R. Carter, K. N. Mendis., manuscript submitted for publication). An understanding of how this cellular downregulation is brought about in endemic patients without compromising antiparasite immunity which also develops with increasing exposure to malaria might provide an important clue to malarial immunity.

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