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Polyclonal Expansion of Peripheral γδ T Cells in Human *Plasmodium falciparum* Malaria

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***Plasmodium falciparum* malaria in humans is associated with an increase in the percentage and absolute number of γδ T cells in the peripheral blood. This increase begins during the acute infection phase and persists for at least 4 weeks during convalescence. In the present study, 25 to 30% of the γδ T cells expressed HLA-DR antigens in vivo and in some patients they proliferated in response to further stimulation by purified human interleukin 2 in vitro. However, there was no in vitro proliferative response to various malarial antigens, including a 75-kDa heat shock protein and a 72-kDa glucose-regulated protein of *P. falciparum* during the acute infection phase. Cytofluorographic studies showed that although an increase of Vδ1$^-$ γδ T cells was largely responsible for the expansion of the total number of γδ T cells, there was also a proportional increase in Vδ1$^+$ cells. These results were confirmed with anchored PCR and by DNA sequencing to characterize at the molecular level the set of T-cell receptor (TCR) δ mRNAs expressed in the peripheral blood of two patients with high levels of γδ T cells. In each case, most of the TCR δ mRNA transcripts corresponded to nonproductively rearranged δ genes (unrearranged Jδ or near Jδ spliced to Cδ). In those sequences which did represent productively rearranged genes, most of the transcripts originated from a Vδ2/Jδ1 joining, as in normal individuals. A minority of transcripts originated from a Vδ1/Jδ1 rearrangement, and one originated from a Vα4/Jδ1 rearrangement. Polyclonal activation of γδ T cells was inferred from the extensive junctional diversity seen in the δ mRNAs analyzed. Expansion of a heterogeneous set of both Vδ1$^-$- and Vδ1$^+$-bearing T cells suggests that the elevated levels of γδ T cells seen during acute *P. falciparum* malaria arose from immune responses to multiple distinct parasite antigens or unidentified host factors.**

T lymphocytes express two different types of antigen receptors (T-cell receptors [TCR]) in a molecular complex with CD3 surface proteins (7, 37). Most mature T lymphocytes express the αβ TCR, which recognizes antigens in association with self major histocompatibility complex (MHC) antigens (12) and is responsible for both regulatory functions and cell-mediated immune responses. A small subpopulation of T cells express the γδ TCR, which can recognize an antigen in an MHC-restricted or non-MHC-restricted fashion (46), but the role of γδ T cells in the immune response is not well understood. As with genes for the αβ TCR, γδ TCR genes undergo V(D)J gene rearrangements during T-cell ontogeny (50). Although the germ line diversity of the γδ genes is limited by a relatively small number of V, D, and J gene segments (32), extensive junctional diversity can be generated because of imprecise joining, N-region diversity, and the tandem use of Dδ gene segments (23, 31). Since the junctional region of the TCR is believed to be the contact site for nominal antigens (12), the extensive junctional diversity of the γ, and particularly the δ, chain suggests that the γδ TCR has the potential to interact with a large number of different ligands.

Although γδ T cells can produce lymphokines (43) and act as both MHC-restricted (11) and non-MHC-restricted (5, 8) cytotoxic effector cells, the nature of the antigens recognized, as well as the process of antigen recognition by γδ T cells, is not clearly understood. Most of the information on γδ T-cell reactivity to microbial antigens has been generated from studies on the response of these cells to mycobacterial antigens, especially mycobacterial heat shock proteins (reviewed in reference 45). Although specificity to mycobacterial Hsp65 was reported in rheumatoid arthritis synovial fluid (26) and the peripheral blood mononuclear cells (PBMC) of purified protein derivative-reactive individuals (22), further evidence shows that most γδ T cells responding to mycobacterial antigens do not react to intact Hsp65 (29). The main antigenic components of *Mycobacterium tuberculosis* that stimulate human γδ T cells appear to consist of protease-resistant ligands, suggesting that they are nonprotein in nature (44). Furthermore, the γδ T-cell response is limited almost exclusively to Vγ9/Vδ2-bearing cells but the junctional sequences of these cells are diverse (42). In unexposed individuals, up to 50% of the peripheral γδ T cells respond to mycobacterial antigens in an in vitro proliferative assay (29). Together, these findings suggest that mycobacterial antigens activate γδ T cells in a superantigen-like fashion; i.e., all T cells expressing a given V region respond, irrespective of their unique junctional sequences. It is not clear whether both Vγ9 and Vδ2 are required for reactivity, since Vγ9 and Vδ2 are coexpressed in most peripheral γδ T cells (6, 10, 17).

Elevated levels of γδ T cells have been detected in several other infections, including the protozoan infections cutaneous

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leishmaniasis (39), trypanosomiasis (38), malaria (25, 47), and toxoplasmosis (13). The antigen specificity and function of γδ T cells in these infections are unknown. We have previously reported that the elevated level of γδ T cells in the peripheral blood of patients acutely infected with *Plasmodium falciparum* is present for at least 4 weeks after the start of antimalarial therapy (25) and in fact may persist for months (46a). γδ T cells may have an important role in the immune response against *P. falciparum* by functioning as non-MHC-restricted cytotoxic cells directed towards intraerythrocytic parasites. On the other hand, it has been suggested that lymphokines produced by activated γδ T cells could induce the production of inflammatory cytokines, such as tumor necrosis factor alpha and interleukin 6 (IL-6), and thus contribute to the immunopathology of severe malaria (34).

To further characterize the γδ T-cell response in patients with acute *P. falciparum* malaria, we determined the phenotypes of the expanded γδ T-cell population and their response to malaria-specific antigens. We also used anchored PCR (A-PCR) and DNA sequencing to assess the repertoire of Vδ genes expressed by T lymphocytes of acutely infected patients.

MATERIALS AND METHODS

Study population. We studied adult patients with acute *P. falciparum* malaria admitted to the Hospital for Tropical Diseases, Bangkok, Thailand. All of the patients had acquired their infections either in Chantaburi Province, eastern Thailand, or Kanchanaburi Province, western Thailand. The transmission in both locations is of the focal, "forest fringe" type, affecting mainly young migrant workers from areas where the disease is nonendemic. Most of the patients gave a history of a few malaria attacks, while some were experiencing their first clinical infections. Twenty-two subjects who had never experienced malaria were recruited as controls. Informed consent was obtained from all of the individuals. The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Clinical studies. *P. falciparum* infection was confirmed by detection of asexual forms of the parasites in peripheral blood smears. Parasite density was determined by counting the number of asexual forms of *P. falciparum* per 1,000 erythrocytes or per 200 leukocytes. Baseline laboratory investigations included complete blood count, blood urea nitrogen, serum creatinine, total bilirubin, and aspartate aminotransferase. Urine was tested to determine the presence of sulfa and 4-aminoquinoline antimalarial agents. Patients were treated with either a single dose of mefloquine or a combination of quinine and tetracycline for 7 days. All of the patients remained in the hospital for the duration of the study.

Antigens. Three malaria antigens were prepared by using standard methods which have been well described in the literature. None of the preparations had any toxic effects on T lymphocytes. A soluble malaria antigen was prepared from an isolate of *P. falciparum* (C129/84) collected in Thailand (24). The antigen was used at an optimal concentration of 15 μg/ml. A crude schizont lysate was prepared from trophozoite- or schizont-infected erythrocytes enriched on a Percoll gradient (2). The lysate was used at concentrations of 10 to 40 μg/ml. Infected erythrocytes (IRBC) containing trophozoites and schizonts taken directly from nonenriched cultures with 3 to 5% parasitemia were cocultured with PBMC at a density of 10^4 to 10^6 IRBC per 10^6 PBMC (20). Uninfected erythrocytes from the same donor were used as a control. Two fusion proteins of *P. falciparum*, a 75-kDa heat shock-related protein and a 72-kDa glucose-regulated protein (33), were kind gifts of Nirbhay Kumar, Johns Hopkins University, Baltimore, Md. These proteins were used at the optimal concentration of 25 μg/ml. Purified human IL-2, purchased from Electro-Nucleonics Inc., Silver Spring, Md., was used at a concentration of 64 U/ml. All reagents were stored at −20°C until used.

Isolation of mononuclear cells. PBMC were obtained by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) sedimentation of heparinized venous blood taken from patients on admission (day 0) and on days 7 and 28 after the start of treatment. The cells were washed three times with Hanks balanced salt solution (Flow Laboratories, Ayreshire, Scotland) and suspended at 10^6/ml in RPMI 1640 (Flow Laboratories) containing 10% heat-inactivated human AB serum, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (Sigma, St. Louis, Mo.), 100 U of penicillin per ml, 100 μg of streptomycin per ml (GIBCO, Grand Island, N.Y.), and 2 mM L-glutamine (Sigma).

Phenotypic analysis. To determine the kinetics of the γδ T-cell response in vivo, 10^6 freshly isolated PBMC were directly stained with fluorescein isothiocyanate (FITC)-conjugated anti-Cδ (TCRδ1; T Cell Sciences, Cambridge, Mass.) or anti-Vδ1-Jδ1/2 (δTCS-1; T Cell Sciences) in accordance with the manufacturer's instructions on each of the study days. PBMC were also double stained with FITC-conjugated TCRδ1 and phycoerythrin (PE)-conjugated anti-CD8 (Leu2a; Becton Dickinson, Mountain View, Calif.) or PE-conjugated anti-HLA-DR (Becton Dickinson) to determine the proportion of $CD8^+$ γδ T cells and the proportion of γδ T cells which was activated in vivo. Control antibodies included isotype-matched, irrelevant PE-conjugated and FITC-conjugated mouse myeloma antibodies (control immunoglobulin G1-PE and immunoglobulin G1-FITC; Becton Dickinson).

Ten thousand cells from each cell preparation were analyzed by fluorescence-activated cell sorting with a FACSCAN apparatus (Becton Dickinson). Forward angle and 90° light scatter patterns were used to gate on the lymphocyte population. The fluorescent intensities of control and test cells were analyzed with Consort C30 software (Becton Dickinson). The proportion of positive cells, expressed as a percentage of total PBMC, was obtained from both histograms and two-parameter cytogram analysis. Absolute numbers of total $\gamma\delta^+$ T cells and subsets were calculated by using the total leukocyte count and percentage of lymphocytes and the proportions of PBMC positive for the different markers.

To determine the antigen specificity of the γδ T cells in *P. falciparum* malaria, PBMC were cultured at 10^6 per well in 24-well Linbro plates (Flow Laboratories) with various malaria antigen preparations and purified human IL-2. Unstimulated cells incubated in medium alone served as controls. At the end of 6 to 7 days of incubation, cells were harvested and double stained with PE-conjugated anti-IL-2 receptor (anti-CD25; Becton Dickinson) and FITC-conjugated anti-CD4 (Leu3; Becton Dickinson) or TCRδ1. Fluorescence-activated cell sorter analysis of cells stimulated with IL-2 was performed with the gate set on large blastic lymphocytes. The proportion of blasts was expressed as a percentage of the whole lymphocyte population. The proportions of $CD4^+$, γδ, and $V\delta1^+$ T cells within the blastic population were also determined.

PCR amplification and DNA cloning and sequencing. Total cellular RNA was prepared by the Nonidet P-40 lysis method with PBMC from 20 ml of whole blood obtained on day 7. First-strand cDNA was synthesized from total RNA by the method of Gubler and Hoffman (21) with some modifications. Briefly, 25 μg of total RNA was mixed with 5 μg of oligo(dT) in a reaction mixture containing each deoxynucleoside triphosphate at 1 mM, 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; New

TABLE 1. Percentages and absolute numbers of γδ T cells in peripheral blood of controls and patients with acute *P. falciparum* malaria[a]

Cell type	Mean % (mean absolute no.) of cells ± SD in PBMC of:			
	Controls (n = 22)	Patients (n = 22)		
		Day 0	Day 7	Day 28
$\gamma\delta^+$	5.0 ± 2.6 (115 ± 60)	9.5 ± 7.8[b] (125 ± 103)	16.4 ± 7.0[c] (450 ± 192)	17.9 ± 7.32[c] (558 ± 227)
$V\delta1^+$	1.0 ± 0.4 (23 ± 9)	1.5 ± 1.9 (20 ± 25)	3.8 ± 2.8[c] (104 ± 77)	3.8 ± 3.1[c] (104 ± 85)
$CD8^+/\gamma\delta^+$	0.9 ± 0.9 (21 ± 21)	1.0 ± 1.0 (13 ± 13)	2.1 ± 1.1[d] (58 ± 30)	2.2 ± 0.6[c] (69 ± 19)
$DR^+/\gamma\delta^+$	1.4 ± 1.4 (34 ± 32)	1.6 ± 1.3 (21 ± 17)	4.9 ± 4.7[b] (134 ± 129)	4.1 ± 2.5[d] (128 ± 78)

[a] PBMC were freshly isolated from patients on admission (day 0) and on days 7 and 28 after the start of treatment. Cells (10^6) were stained with monoclonal antibodies for total γδ T cells, $V\delta1^+$ T cells, $\gamma\delta^+/CD8^+$ cells, and $\gamma\delta^+/DR^+$ cells. Stained cells were analyzed with a FACSCAN apparatus. The proportion of positive cells was expressed as a percentage of the total PBMC count. The absolute numbers were calculated as follows: total leukocyte count × % lymphocytes × % surface antigen-positive PBMC. PBMC from normal controls were studied similarly.

[b] $P < 0.05$ compared with controls.

[c] $P < 0.001$ compared with controls.

[d] $P < 0.005$ compared with controls.

England Nuclear, Boston, Mass.), 100 U of rRNasin, and 60 U of reverse transcriptase (Seikagaku America Inc., St. Petersburg, Fla.) in 50 mM Tris (pH 8.8)–50 mM KCl–60 mM $MgCl_2$–10 mM dithiothreitol in a total reaction volume of 100 μl. The solution was incubated at 42°C for 90 min. The cDNA was purified by being passed through a 200/400-mesh Bio-Gel column (4 by 300 mm; Bio-Rad, Richmond, Calif.) prepared in 1 mM Tris-HCl (pH 8.0)–0.1 mM EDTA. The radiolabelled cDNA was collected from the column in 25-μl fractions. The first two fractions of cDNA were pooled, tailed with dGTP by using terminal transferase (Boehringer Mannheim, Laval, Quebec, Canada), extracted with phenol-chloroform, and chloroform and ethanol precipitated. The dGTP-tailed cDNA was suspended in 50 μl of water, and 0.2 to 1.0 μl was used as a PCR template.

A-PCR (35) was performed on the cDNA template in a reaction mixture containing each primer at 1 μM, each deoxynucleoside triphosphate at 250 μM, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 2 U of *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.) in a final volume of 50 μl. The 5′ primer was 5′-CTATCTAGAGAGCTCGCGGCCG(C_{14})-3′ (referred to as AnPC), and the 3′ primer was 5′-GACAAGCGACATTTGT TCC-3′. The reaction was set to denature at 94°C for 1 min, anneal at 50°C for 1 min, and extend at 72°C for 1 min for a total of 30 cycles. The PCR product was analyzed by electrophoresis on a 1.2% agarose gel. The band was excised, recovered with Gene Clean (Bio 101, Inc., La Jolla, Calif.), and treated with the Klenow fragment of DNA polymerase I. The blunted product was digested with *Not*I (this site is present in the AnPC primer) to facilitate cloning into the *Not*I-*Eco*RV-digested pBluescript KS− vector (Stratagene Inc., La Jolla, Calif.). Plasmid DNA from the recombinant clones was prepared by standard methods, and the double-stranded DNA template was sequenced with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). A nested Cδ oligonucleotide (5′-GGTTTGGTATGAGGCTGAC-3′) was used as a sequencing primer.

Statistical analysis. Group means were compared with Student's t test, and correlation was assessed by Spearman's rank test.

RESULTS

Kinetics of γδ cell response. Twenty-two patients with confirmed *P. falciparum* malaria were studied longitudinally to determine the kinetics of the γδ T-cell response in vivo. The results are summarized in Table 1. The mean percentage of γδ T cells, but not the mean absolute number, was significantly elevated on admission compared with that of controls ($P < 0.05$). Both the mean percentage and absolute number became more markedly elevated on days 7 and 28 after the start of antimalarial therapy. No difference was observed in the γδ T-cell responses of patients treated with either a single dose of mefloquine or the combination of quinine and tetracycline for 7 days (data not shown). Twenty-five to 30% of γδ T cells on days 7 and 28 expressed the HLA-DR antigen, indicating that these cells were activated in vivo.

To determine whether there were any clinical features which may be positively correlated with the magnitude of the γδ T-cell response on admission, some related factors were examined. Seven patients had received or self-administered subtherapeutic doses of antimalarial drugs before admission. Although these patients tended to have higher levels of γδ T cells on admission (mean ± standard deviation, 13.1% ± 8.8%), the difference between them and those of 15 untreated patients (8.2% ± 6.0%) was not statistically significant ($P > 0.10$). There was no correlation between parasitemia on admission and the percentage of γδ T cells ($r = 0.148$; $P > 0.50$). There was also no significant difference between 13 patients with uncomplicated malaria (11.3% ± 8.9%) and 9 patients with severe (6.3% ± 6.8%) malaria ($P > 0.10$), as defined by hyperparasitemia (>5.0%), the occurrence of cerebral complications, and/or biochemical evidence of kidney or liver dysfunction (serum creatinine of >200 μmol/liter or bilirubin of >50 μmol/ml and aspartate aminotransferase activity of >50 U/liter).

γδ T-cell subsets. By using monoclonal antibody δTCS-1, which recognizes Vδ1 gene products and reacts with cells displaying either a Vδ1/Jδ1 or Vδ1/Jδ2 rearrangement, it was demonstrated that although there was a marked increase of $V\delta1^-$ cells, there was a proportional expansion of the $V\delta1^+$ subset (Table 1). There was also a significant increase in γδ T cells which were $CD8^+$. In contrast to an early rise in the mean percentage of total γδ T cells, the increase in $V\delta1^+$ and $CD8^+$ cells was first evident on day 7.

Stimulation with malarial antigens. PBMC isolated from six patients were incubated with optimal concentrations of soluble malaria antigen, schizont lysate, IRBC, and the *P. falciparum* 75-kDa heat shock-related and 72-kDa glucose-regulated proteins for 6 to 7 days. IL-2 receptor expression was used as an indicator of in vitro activation. Cytofluorographic analysis of cells obtained on days 0 and 7 of hospitalization revealed no increase in activated T cells in response to any of the antigens, compared with unstimulated cells (data not shown).

TABLE 2. Response of peripheral CD4$^+$ and γδ T cells to stimulation by different *P. falciparum* antigens during convalescence[a]

Antigen	Mean % (± SD) of cells that were:			
	CD4$^+$	CD4$^+$/CD25$^+$	γδ$^+$	γδ$^+$/CD25$^+$
None	45 ± 6	3 ± 2	7 ± 3	0.2 ± 0.1
Soluble malaria antigen	48 ± 7	11 ± 1[b]	8 ± 3	0.1 ± 0.1
Pfhsp[e]	47 ± 4	8 ± 2[b]	6 ± 2	0.2 ± 0.1
Pfgrp[f]	46 ± 4	7 ± 2[b]	6 ± 2	0.3 ± 0.1
Schizont lysate	51 ± 3	21 ± 8[c]	7 ± 2	0.1 ± 0.1
IRBC	42 ± 8	21 ± 7[c]	14 ± 7	7.0 ± 6.2[d]

[a] PBMC harvested from six patients on day 28 of hospitalization were incubated with various malarial antigen preparations for 6 to 7 days. The cells were then harvested and analyzed for CD4$^+$/IL-2-R$^+$ cells and γδ$^+$/IL-2-R$^+$ cells. The proportion of double-positive cells, expressed as a percentage of the total PBMC count, was compared with that of unstimulated controls.
[b] $P < 0.01$ compared with controls.
[c] $P < 0.001$ compared with controls.
[d] $P < 0.025$ compared with controls.
[e] Pfhsp, *P. falciparum* 75-kDa heat shock-related protein.
[f] Pfgrp, *P. falciparum* 72-kDa glucose-regulated protein.

TABLE 3. Response of peripheral CD4$^+$ and γδ T cells to stimulation by purified human IL-2[a]

Cell source and day after treatment started	% of cells that were:			
	Blasts	CD4$^+$	γδ$^+$	Vδ1$^+$
Patient 1				
7	51	6	52	38
28	17	17	22	13
Patient 2				
7	74	10	45	25
28	27	20	17	10
Patient 3				
7	40	12	40	18
28	15	18	9	5

[a] PBMC harvested on days 7 and 28 of hospitalization were incubated with 64 U of purified human IL-2 per ml. Cells were harvested after 6 to 7 days of incubation and analyzed for total γδ T cells and Vδ1$^+$ cells. The proportion of blastic cells (>98% CD25$^+$) was expressed as a percentage of the whole lymphocyte population. The percentages of CD4$^+$ T cells, γδ T cells, and Vδ1$^+$ cells within the blastic population were determined. Unstimulated cultures contained less than 5% blastic cells.

The responses of PBMC isolated on day 28 are summarized in Table 2. In unstimulated cultures, the mean percentage of γδ T cells was 7%, compared with a baseline (before-stimulation) value of 16.4%, in these six patients. Activation of CD4$^+$ T cells by all of the antigen preparations was seen, while a significant increase in the percentage of activated TCRδ1$^+$ T cells occurred when PBMC were stimulated by IRBC but not when they were stimulated by control erythrocytes ($P < 0.05$).

Expansion of γδ T cells and Vδ1$^+$ cells after incubation with IL-2. In contrast to the slight response to a specific antigen, PBMC obtained from three of the six patients on day 7 of hospitalization showed a dramatic response to IL-2 (Table 3). Large, blastic lymphocytes, >98% of which were CD25$^+$, made up 40 to 74% of the total lymphocyte population, 40 to 50% of which were TCRδ1$^+$. There was an increase of both the Vδ1$^+$ and Vδ1$^-$ subsets. The mean number of cells per well at the end of the incubation was 1.9×10^6, compared with 0.55×10^6 for unstimulated cultures. These three patients had uncomplicated *P. falciparum* malaria and were experiencing their first infections, as far as could be ascertained. The response to IL-2 was much reduced when the same patients were restudied on day 28.

Molecular analysis of TCR δ expression. A-PCR was used to analyze the δ transcripts present in PBMC of two patients with acute *P. falciparum* malaria who had high levels of γδ T cells (28 and 29% of PBMC), and the results are shown in Fig. 1 and 2. In both cases, most (80%) of the TCR δ arose from nonproductively rearranged δ genes (Fig. 1). Three different types of transcripts were identified: (i) germ line Jδ1 spliced to Cδ, (ii) germ line Jδ2 spliced to Cδ, and (iii) a sequence upstream of Jδ1 spliced to Cδ.

A smaller percentage (20%) of δ transcripts arose from productively arranged genes (i.e., Vδ-Dδ-Jδ joined), and these sequences are shown in Fig. 2 and Table 4. In agreement with the phenotypic study, most of the transcripts arose from Vδ2, while a relatively small portion utilized Vδ1. Preferential usage of Jδ1 was seen in the transcripts from patient 20. Extensive diversity was observed in the junctional regions, and most of the δ transcripts analyzed encoded unique junctional segments. One sequence (23-189) corresponded to a rearrangement of Vα4.1/Dδ3/Jδ1, which apparently formed a functional δ chain. Surprisingly, the deduced amino acid sequences (Fig. 2) showed that every one of the transcripts analyzed corresponded to an in-frame joining event (i.e., a transcript that encodes a functional TCR δ chain).

DISCUSSION

Since the first description in 1986 (7), extensive progress has been made in the molecular characterization of both the proteins and the genes of the γδ TCR. Studies aimed at determining antigen specificity and function have been less conclusive. Although elevated levels of γδ T cells have been reported for many infections (1, 13, 14, 25, 38, 39, 42, 47, 51,

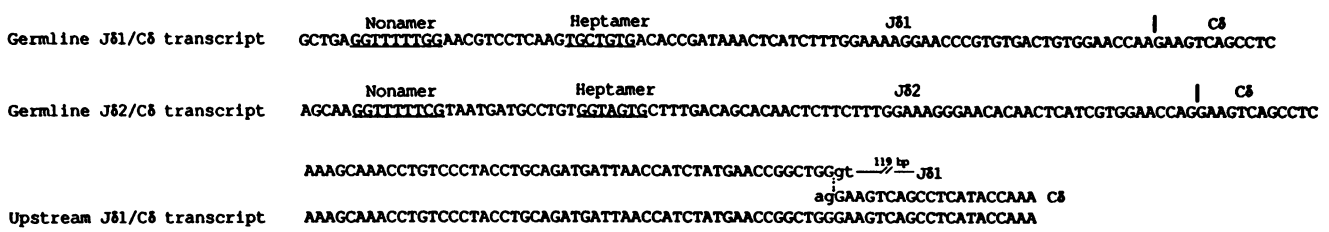

FIG. 1. Sequences of unproductively rearranged TCR δ transcripts found in controls and patients with malaria. Jδ1/Cδ and Jδ2/Cδ transcripts contain the conserved heptamer-nonamer sequence, indicating that they are transcribed from the unrearranged gene. The upstream Jδ1/Cδ transcript contains the germ line sequence 119 bp upstream of Jδ1 joined to Cδ by an alternative splicing mechanism. The splice sites are indicated by the lowercase letters and the broken line.

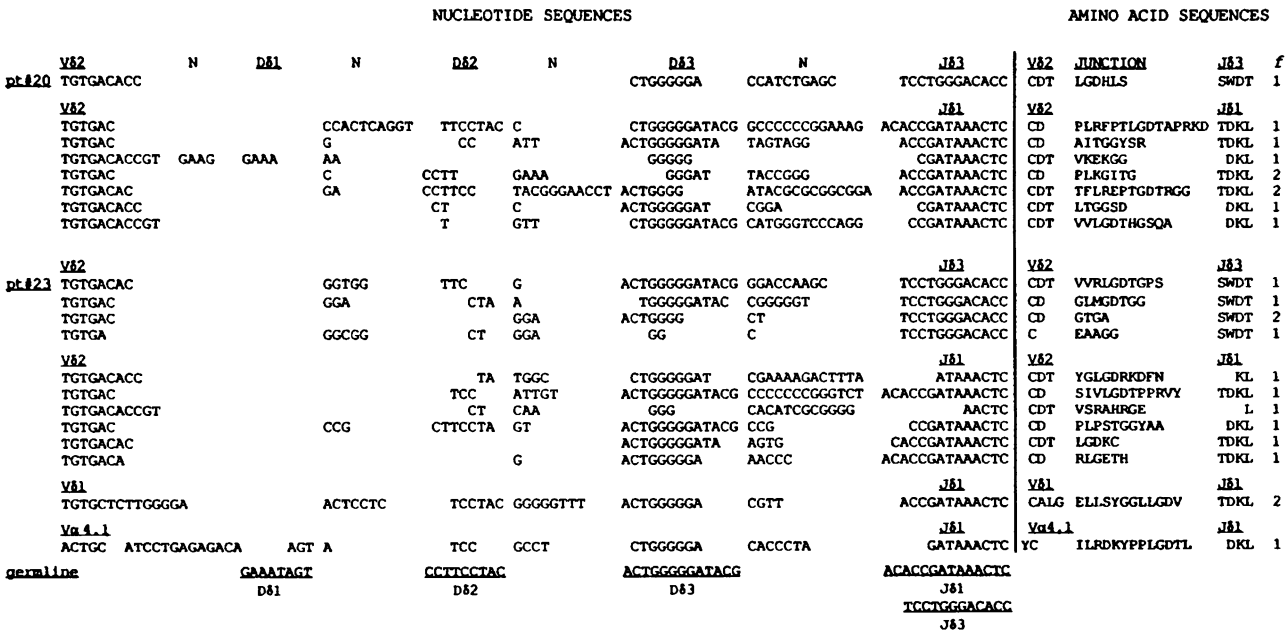

FIG. 2. TCR δ junctional sequences derived from PBMC of two patients (pt) with acute *P. falciparum* malaria. The A-PCR products generated from cDNA were cloned and sequenced by the dideoxy-chain termination method. Deduced amino acid sequences are shown in the single-letter code. The germ line sequences of Dδ1, Dδ2, Dδ3, Jδ1, and Jδ3 are underlined. The template-independent (N) sequences at the junctions are indicated, and the numbers of individual members in each sequence are shown in column *f*.

55), as well as disorders associated with immune abnormalities (3, 9, 18, 36, 49, 52, 53, 56), the nature of the antigens recognized by them is not clear, nor is the process by which these antigens are processed and presented.

In the present study, we extended our previous findings on the γδ T-cell response in the peripheral blood of patients with acute *P. falciparum* malaria. Levels of γδ T cells at the time of presentation did not appear to correlate with any clinical or laboratory parameters. The normal mean absolute number on day 0 implies that the increased percentage during this stage of the illness was due to a decrease of αβ T cells, as we have previously shown (24). However, by day 7, elevated absolute numbers were established in all 22 patients. A proportion of the expanded γδ T cells expressed HLA-DR antigens, which suggests that the cells were activated in vivo. The expansion involved both major subsets of γδ T cells in the adult peripheral blood, viz., both Vδ1$^+$ and Vδ1$^-$ cells. The ratio of Vδ1$^+$/Vδ1$^-$ cells therefore remained the same as in normal controls throughout the study period. In 10 additional patients studied since the completion of the longitudinal study, staining with an anti-Vδ2 monoclonal antibody (T Cell Sciences) confirmed that the Vδ1$^-$ cells were indeed Vδ2$^+$, and together the Vδ1$^+$ and Vδ2$^+$ cells accounted for greater than 90% of the total γδ T cells in these patients (23a).

A similar increase of both Vδ1$^+$ and Vδ2$^+$ cells has been observed in granulomatous lesions of *Mycobacterium leprae* in which cells infiltrating the epidermis primarily express Vδ1-encoded TCRs while Vδ2-bearing cells predominate in the dermis and peripheral blood (55). In *P. falciparum* malaria, Vδ1$^+$ T cells may be of splenic origin since most of the γδ T cells in the spleen are Vδ1$^+$ (16). A marked increase of γδ T cells in the splenic red pulp was reported in a patient with *P. falciparum* malaria (40% of the total T cells versus 17% in normal controls) (4). Unfortunately, the subsets of γδ T cells were not analyzed in that study. Similarly, increased CD8$^+$ γδ T cells were seen in the spleens of *Plasmodium chabaudi*-infected mice (38). Whether increased CD8 surface antigen expression has any functional role in vivo is not known. In vitro studies of human T-cell clones suggest that CD8$^+$ γδ T cells are not a distinct functional subset as measured by cytokine production or cytotoxicity (40).

The expansion of both Vδ1$^+$ and Vδ1$^-$ γδ T cells in our patients contrasts with the exclusive increase of Vγ9/Vδ2 γδ T cells in the PBMC of unexposed individuals after stimulation with *P. falciparum* schizont lysate (2), merozoites (17), and IRBC (19) in vitro. The discrepancy suggests that a more complex immune response has been mounted in vivo. In the case of stimulation by merozoites, the estimated precursor frequency of 1 in 150 to 1 in 300 is much higher than expected for antigen-specific responsive cells and is consistent with a superantigen effect of *P. falciparum*. The frequency for IRBC-responding cells (1:2,000 to 1:3,000) was more compatible with antigenic stimulation. However, as other investigators (57) were unable to elicit a γδ T-cell response in vitro with IRBC in PBMC from normal individuals, the response of naive PBMC may depend on the particular antigen preparation or parasite isolate used. In murine salmonellosis, it has been demonstrated that the γδ T-cell response is induced by an avirulent but not by a virulent bacterial strain (15).

TABLE 4. Vδ and Jδ gene segment usage in patients with malaria

Patient no.	% of γδ$^+$ T cells	No. of clones bearing:		Total no. of clones	No. of clones bearing Jδ1, Jδ3		
		Vδ2	Vδ1		Vδ2	Vδ1	Total
20	28	10	0	10	9, 1	0, 0	9, 1
23	29	11	2	13	6, 5	2, 0	8, 5

The specific recognition of malarial antigens by γδ T cells from patients was difficult to demonstrate in vitro. When PBMC obtained from patients on days 0 and 7 were stimulated by malarial antigen preparations, no increase in the percentage of either activated $CD4^+$ or γδ T cells was seen. The antigen preparations included a soluble protein preparation, a schizont lysate, IRBC, and two recombinant heat shock-related proteins of *P. falciparum* which have been shown to stimulate PBMC of immune donors (33). By day 28, there was a $CD4^+$ T-cell response to soluble malaria antigens. The response to a schizont lysate in our patients also appeared to be mediated predominantly by $CD4^+$ T cells. In contrast, there was a concomitant increase in the percentages of activated $CD4^+$ and $\gamma\delta^+$ T cells compared with that of unstimulated PBMC in response to IRBC. Although it has been suggested that in some inflammatory diseases, the increase in γδ T cells may be secondary to activated, IL-2-producing αβ T cells (30), this explanation appears unlikely in view of our results, as activated $CD4^+$ cells in response to the other malaria antigen preparations did not induce a γδ T-cell response.

There are several plausible explanations for the difficulties in demonstrating a proliferative response of γδ T cells to malarial antigens. Malaria-specific γδ T cells may already be activated in vivo and are therefore anergic to further antigen stimulation in vitro. This hypothesis is consistent with the finding that the activated cells were able to respond to further stimulation by exogenous IL-2. The proliferative response to IL-2 was unlikely to be due to nonspecific stimulation of γδ T cells, as it was not observed in PBMC harvested on day 28. It is also conceivable that γδ T cells respond to antigen stimulation by producing cytokines not related to proliferation or displaying other cellular functions, such as cytotoxicity. This has been shown for γδ T cells stimulated by staphylococcal enterotoxin A, which did not proliferate but were cytotoxic against target cells primed with the specific antigen (48). Apoptosis can be triggered in activated γδ T cells, and γδ T-cell proliferation may actually be downregulated by a specific antigen and IL-2 (28). Antigen processing and presentation requirements may also be different for γδ TCR, although γδ T-cell clones responsive to short peptides of tetanus toxin presented by class II MHC molecules have been described (27). Finally, the possibility exists that the expansion of γδ T cells seen in acute *P. falciparum* malaria is not malaria antigen specific but a response to host antigens released during the infection.

To characterize the TCR repertoire of γδ T cells which are induced during *P. falciparum* infection, the TCR δ chain was analyzed by using the A-PCR. With this procedure, the cDNA sequences of a representative sample of all of the δ transcripts which are present in PBMC are obtained. By adding a poly(dG) tail to the first-strand cDNA, PCR amplification can be done with a single specific Cδ primer at the 3′ end and a universal oligo(dC) (AnPC) at the 5′ end. The procedure allows detection of all of the transcripts that carry the Cδ gene segment. Thus, it can be used to analyze Vδ gene usage independently of phenotypic characterization by monoclonal antibodies, and novel V gene usage can be identified directly. Analysis of TCR δ gene transcripts from the patients showed that most of them were unproductive, suggesting that some promoter in the vicinity of Jδ can drive transcription of the unrearranged genes. Among the productively arranged genes, the frequency of Vδ2/Vδ1 gene usage in patient 23 was consistent with results of the phenotypic study, and the absence of Vδ1 transcripts in patient 20 is most likely due to the small sample size. The extensive sequence diversity observed in the junctional regions suggests that a diverse set of TCR δ chains is expressed in these patients and clearly demonstrates that polyclonal activation of γδ T cells has occurred. There is no analysis of the γ chain which would be necessary to address the issue of preferential Vγ usage. However, the data do suggest that it is unlikely that a given γ is selected in the response to *P. falciparum*, as no given δ is selected.

The γδ TCR repertoire is now known for a number of disorders (18, 52, 53, 55). A pattern is emerging which indicates that there are differences in the local as opposed to the systemic γδ T-cell response to microbial and self antigens. γδ T cells isolated from affected sites, e.g., synovial fluid in rheumatoid arthritis, lung tissue in sarcoidosis, skin lesions in leprosy, and nerve lesions in multiple sclerosis all show evidence of clonal expansion of γδ T cells, while PBMC from the same patients display a polyclonal response. In malaria, it can be argued that the peripheral blood is the primary affected "organ," since the parasites are in the circulation system. Nevertheless, it would be of great interest, if it were technically and ethically feasible, to study the clonality of splenic γδ T cells in the human disease, as the spleen is intimately involved in host defense against plasmodia. The functional difference between clonal and polyclonal responses is not clear, as a number of junctionally diverse murine γδ T-cell clones can all respond to mycobacterial Hsp60 and a 17-amino-acid synthetic peptide of the *M. leprae* Hsp60 sequence (41).

The role of γδ T cells in acute malaria is speculative. The hypothesis that these cells contribute to the immunopathology of *P. falciparum* malaria is inconsistent with the kinetics of γδ T-cell activation and expansion reported here and in previous studies (25, 47) which show that the levels remain elevated for weeks during convalescence. Indeed, our current data suggest an inverse relationship between the percentage of γδ T cells and disease severity, although the difference in the mean percentages did not reach statistical significance. On the other hand, preliminary data obtained with a murine model suggest that γδ T cells have a role in protection against malaria (54). Mice lacking functional αβ T cells were able to mount a protective immune response which significantly inhibited the development of the liver stages of *Plasmodium yoelii*. The same effect was obtained when a γδ T-cell clone from a sporozoite-immunized αβ knockout mouse was adoptively transferred to normal animals. The possibility that γδ T cells also inhibit the development of blood stage *P. falciparum* parasites is currently under investigation.

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