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CONTRIBUTION OF HUMORAL IMMUNITY TO THE THERAPEUTIC RESPONSE IN FALCIPARUM MALARIA

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Abstract. The contribution of humoral immunity to the therapeutic response in acute falciparum malaria was assessed in a case-control study. Forty adult Thai patients with acute falciparum malaria who had subsequent recrudescent infections and 40 patients matched for age, therapeutic regimen, and disease severity who were cured by Day 28 were studied. All cured patients had positive immunoglobulin (Ig) G to ring-infected erythrocyte surface antigen (RESA) in their admission plasma, compared with only 60% of patients who failed to respond to treatment ($P < 0.001$). The proportion of IgM-positive cases at admission was also higher in the successfully treated group than in the group with failure (70% versus 30%) ($P < 0.001$). The geometric mean (95% confidence interval) reciprocal IgG titer at admission was significantly higher in cured patients (187.0 [83.5–418.3]) compared with those who experienced treatment failure (11.6 [5.1–26.5]) ($P < 0.001$). The patients with uncomplicated malaria who were both IgG and IgM positive at admission had significantly shorter fever clearance times and lower admission parasitemia levels compared with those who were negative ($P = 0.01$ and $P = 0.02$, respectively). The median (range) *in vitro* parasite multiplication rate was significantly lower in cultures containing positive anti-RESA antibody plasma compared with those containing normal plasma (0.7 [0.1–3.5] versus 2.6 [0.1–12.1]; $P < 0.001$). These results suggest that antimalarial antibodies may play an important supportive role in the therapeutic response to antimalarial drugs during acute falciparum malaria.

INTRODUCTION

It is well known that immunity contributes to the therapeutic response in malaria, with better treatment outcomes in patients with background immunity.[1] In high-transmission areas, spontaneous resolution of malaria is usual in older children and adults. Even in low-transmission areas such as Thailand, background immunity may contribute significantly to the therapeutic response.

The ring-infected erythrocyte surface antigen (RESA/Pf155) is a specific antigen derived from *Plasmodium falciparum* during intraerythrocytic infection. This antigen is deposited in the red blood cell membrane shortly after merozoite invasion and can be detected as membrane staining of ring-stage infected red blood cells by modified immunofluorescence assay.[2–4] Antibodies to RESA have been associated with protection from malaria and shown to inhibit *P. falciparum* growth *in vitro* efficiently.[5–11] For a time, this antigen was considered as a potential vaccine candidate.[12,13] Antibodies to RESA can be used as markers of the broader humoral immune response.[14,15] To assess the contributory role of humoral immunity to the therapeutic response, we compared the time profiles of anti-RESA antibodies (immunoglobulin [Ig] G and IgM) and the titers between patients with acute falciparum malaria who were cured (treatment success) and those who developed subsequent recrudescent infections (treatment failure).

MATERIALS AND METHODS

Patients. Adult Thai patients with acute falciparum malaria who were admitted and remained for 28 days in the Hospital for Tropical Diseases, Bangkok, Thailand, were studied. Plasma samples from patients who developed subsequent recrudescent infections (without reexposure) after antimalarial treatment were matched for age, therapeutic regimen, and disease severity and analyzed with samples from those who were cured. These cured patients were selected from a large series of previously untreated patients entered into prospective studies of antimalarial drug efficacy between November 1998 and April 1999 for whom serial samples were available. The patients' series were selected before antibody assays were performed. None of the patients had mixed infections detected microscopically at admission or underlying diseases, and they had not received previous antimalarial therapy. World Health Organization criteria[16] were used to define severe or uncomplicated malaria. All patients were treated with short-acting antimalarial drugs (artesunate alone or in combination with azithromycin or artemether plus lumefantrine). This investigation was part of comparative studies of antimalarial efficacy approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. All patients gave fully informed consent.

Blood sampling and processing. Serial venous blood samples (2–3 mL from each patient) were collected prospectively in heparinized tubes before and after treatment at admission (Day 0), on Days 7, 14, 21, and 28, and on the day of recrudescence. The plasma was collected, heat inactivated at 56°C for 30 min, and stored at −70°C until use. Air-dried and methanol-fixed monolayer blood slides (parasitemia ~ 5%, mostly ring stages) were prepared from a blood group O patient with acute falciparum malaria and stored at −70°C until use.

Parasite count and routine laboratory tests. Parasite counts were performed at 6 hour intervals in severe and at 12 hour intervals in patients with uncomplicated malaria until parasite clearance and daily thereafter. The counts were expressed as numbers of asexual parasites per microliter of blood and were calculated from the numbers of parasitized cells per 1,000 erythrocytes in a thin film or per 200 leukocytes in a thick film stained with Field's stain. Routine hematological and biochemical tests were performed at ad-

mission and were repeated daily for 1 week and then weekly thereafter for severely ill patients, and repeated weekly only for uncomplicated cases.

Antibody assays. Indirect immunofluorescence assays were performed for detection of anti-RESA antibodies as described previously.[2,4] Briefly, 10 μL of diluted plasma in phosphate-buffered saline (PBS) from studied patients and from a malaria-negative healthy Thai donor (as a negative control) were placed on demarcated areas of air-dried and fixed monolayer blood slides, incubated in a humidified chamber for 30 min at room temperature, and washed twice in PBS. Then 5 μL of affinity purified rabbit antihuman IgG (γ-chain specific) or IgM (μ-chain specific) (Dako A/S, Copenhagen, Denmark) conjugated with fluorescein isothiocyanate was added to the previously stained areas on the slides and incubated for 30 min under the same conditions as above. After washing, the slides were mounted with 50% glycerol in PBS containing 10 μg/mL ethidium bromide to visualize the intraerythrocytic parasites. The stained slides were then examined with an ultraviolet light microscope at ×1,000 magnification. Plasma samples were tested initially at 1:50 dilution, and if the tests were negative, they were repeated at 1:5 dilution as the cutoff point of positivity or negativity. To compare the antibody titers between the two groups of patients (cured and treatment failure), antibody titration was established from the admission samples for specific IgG antibodies by use of serial 5-fold dilution from 1:5 to 1:15,625. The assays were performed in a blinded fashion by personnel without knowledge of the microscopic and clinical results of the patients.

Effects of plasma containing anti-RESA antibodies on parasite multiplication inhibition *in vitro*. In order to study the effect of plasma from the patients with positive admission antibodies to RESA on parasite multiplication, plasma from these patients were pooled and incorporated *in vitro* cultures and compared with normal human plasma as controls.[17] The malaria culture media containing 1:5 (v/v) of pooled plasma from studied patients or plasma from a malaria-negative healthy donor (control) were used in cultures for one falciparum laboratory strain (TM267R) and 20 falciparum isolates collected from 6 patients with severe and 14 with uncomplicated malaria. The cultures were started with synchronous ring-form parasites. After one cycle (48 hr), the multiplication rate (defined as the ratio of final parasitemia percentage to initial parasite percentage) was assessed and compared between parasites cultured in plasma from studied patients and those in normal plasma. Parasite counts were performed in a blinded fashion.

Statistical analysis. Data were analyzed by SPSS 8.0 for Windows computer software (SPSS, Chicago, IL). Normally distributed data were compared by Student's *t*-test and nonnormally distributed parameters were compared with the Mann-Whitney *U*-test or log transformed and then compared by Student's *t*-test. Correlation was assessed by Pearson's method for normally distributed data and Spearman's method for nonnormally distributed data. The chi-square test with Yates' correction or Fisher's test was used for comparison of groups.

RESULTS

A total of 80 patients were studied, comprising 40 patients who were cured and 40 patients whose infections later recrudesced (Table 1). Of these, 26 patients (13 with treatment success and 13 with treatment failure) had severe falciparum malaria, and 54 patients (27 with treatment success and 27 with treatment failure) had uncomplicated falciparum malaria. Patients with severe malaria had significantly higher admission parasite levels compared with those with uncomplicated infection ($P < 0.001$). Overall, 35% (28 of 80) of patients had a history of having at least one malaria attack. The admission parasite density was significantly lower in the group with previous malaria attacks compared with the group with primary infections ($P = 0.02$). Within each group of patients, admission parasite counts and other laboratory data were not significantly different between patients with and without recrudescent infections (Table 1). A summary of demographic and clinical data of the patients at admission is shown in Table 2. The overall mean (standard deviation) times to *P. falciparum* recrudescence onset in the treatment failure groups were 21 (2.9) days for severe and 17 (3.4) days for uncomplicated cases ($P = 0.001$). Parasite density, and parasite or fever clearance times at recrudescence did not significantly differ between patients with severe and uncomplicated malaria. Of all patients, 8 (10%) developed subsequent vivax parasitemias Days 7–23 after treatment.

Antibody profiles and seropositivity rate at admission. With positive antibody defined as a titer of ≥ 1:5, all patients with treatment success from the severe (13 of 13) and uncomplicated (27 of 27) malaria groups had consistently positive IgG to RESA from Day 0 until Day 28. In contrast, only 38% (5 of 13) of severe and 70% (19 of 27) of uncomplicated malaria patients, whose infections recrudesced subsequently, had positive IgG at admission samples (Table 2) ($P = 0.002$, and 0.004 respectively). However, all the subsequent samples (from Day 7 to day of recrudescence) of these patients had positive IgG. There was no difference in the proportion of patients with admission positive IgG between the severe and uncomplicated malaria groups ($P = 0.09$).

In contrast to IgG, not all patients with treatment success had positive IgM to RESA at presentation. Overall, 70% (28 of 40) of the cured patients and 30% (12 of 40) of the patients with subsequent recrudescent infections had positive IgM to RESA at admission ($P < 0.001$). In the patients with uncomplicated malaria, the proportion of positive IgM patients was higher in the cured patients (78%) (21 of 27) compared with those with treatment failure (37%) (10 of 27) ($P = 0.004$), but there was no difference within the severe malaria group. All except 2 patients, who were IgM negative at admission, became IgM positive on follow-up days (either by Day 7 or Day 14). The proportion of positive IgM tests at admission was also significantly higher in the patients with uncomplicated (31 of 54, 57%) than in severe (9 of 26, 34.6%) malaria ($P = 0.04$).

Antibody titers. The geometric mean (95% confidence interval) reciprocal IgG titer to RESA at admission was significantly higher in cured patients compared with those with treatment failures both in the severe (141.5 [41.7–479.8] versus 3.4 [1.1–13.7]; $P < 0.001$) and uncomplicated (213.7 [71.9–634.7] versus 20.9 [7.6–57.0]; $P = 0.002$) malaria groups (Figure 1). The patients with a history of previous malaria had also higher antibody titers compared with those whose infections were primary (176.5 [62.5–498.3] versus

TABLE 1
Laboratory baseline data of patients with severe and uncomplicated malaria*

Variable	Severe malaria (n = 26)			Uncomplicated malaria (n = 54)		
	Cure (n = 13)	Treatment failure (n = 13)	Total (n = 26)	Cure (n = 27)	Treatment failure (n = 27)	Total (n = 54)
Parasitemia (parasites/μL), geometric mean (95% confidence interval)	127,673 (53,753–303,179)	98,243 (39,391–244,963)	112,124 (62,460–201,326)	18,264 (11,690–28,543)	28,048 (14,771–53,260)	22,542 (15,424–32,946)
Hematocrit (%)	34.1 (7.9)	33.4 (10.2)	33.8 (8.9)	33.6 (7.0)	38.0 (6.5)	35.8 (7.0)
Total bilirubin (mg %)	3.5 (1.0–21.5)	4.1 (1.0–26.3)	4.0 (1.0–26.3)	1.3 (0.6–7.5)	1.2 (0.5–6.9)	1.3 (0.5–7.5)
Alkaline phosphatase (U/L)	107.6 (42.0)	146.2 (73.2)	126.9 (61.7)	113.6 (58.6)	104.3 (37.8)	108.9 (49.0)
Serum glutamic oxaloacetic transaminase (U/L)†	47.0 (17.0–351.0)	81.0 (37.0–315.0)	57.0 (17–351)	44.0 (12.0–203)	39.0 (21.0–164)	42.5 (12.0–203)
Serum glutamic pyruvic transaminase (U/L)†	27.0 (10.0–82.0)	41.0 (17.0–266.0)	35.5 (10–266)	45.5 (15.0–165)	42.0 (5.0–133.0)	43.0 (5.0–165)
Serum creatinine (mg %)	0.9 (0.2)	1.5 (0.6)	1.2 (0.6)	0.9 (0.1)	1.0 (0.2)	0.9 (0.17)
Blood urea nitrogen (mg %)	26.6 (19.3)	40.0 (25.4)	33.2 (14.4)	13.5 (3.6)	15.3 (4.7)	14.4 (2.2)

* All values are expressed as mean (standard deviation) unless otherwise indicated.
† Median (range).

22.8 [10.5–49.2]; $P = 0.002$). The antibody titers were lower in the patients with severe malaria (22.1 [7.0–69.5]) compared with those with uncomplicated disease (66.8 [30.5–146.4]), but the difference was not statistically significant ($P > 0.05$).

Correlates of anti-RESA antibodies. In uncomplicated malaria, the fever clearance times were significantly longer in patients who were IgG or IgM negative at admission compared with those who were IgG or IgM positive ($P = 0.03$ and $P = 0.008$, respectively). In addition, the patients who were both IgG and IgM negative at admission also had longer fever clearance times compared with those with both IgG and IgM positive ($P = 0.01$). Overall, there was a significant negative correlation between admission reciprocal antibody titers and fever clearance times ($r = -0.56$; $P < 0.001$). This correlation was significant within the uncomplicated malaria group, but not in the severe group. The admission parasite density was significantly higher in the patients who were IgG or IgM negative compared with those who were IgG or IgM positive in patients with uncomplicated malaria ($P = 0.02$ and $P = 0.006$, respectively), but not those with severe malaria. There was no significant difference in parasite clearance times between the patients who were IgG or IgM positive and those who were negative for both severe and uncomplicated malaria groups.

Effects of plasma containing anti-RESA antibodies on parasite multiplication. Starting at the same parasitemias (~1%) in ring-stage cultures, the parasitemia levels after schizogony were compared between culture media containing normal plasma (from malaria-negative healthy donors) and plasma from the patients with positive RESA antibodies. The median (range) multiplication rate of parasites cultured in the media containing positive antibodies was significantly lower than those cultured in media containing normal plasma from the malaria negative healthy donors (0.7 [0.1–3.5] versus 2.6 [0.1–12.1], $P < 0.001$) (Figure 2).

DISCUSSION

In this case-control study, the proportion of patients with positive anti-RESA antibodies and the geometric mean antibody titers at admission were significantly higher in patients whose malaria was cured compared with those who experienced treatment failure despite similar antimalarial treatment regimes. This suggests a supportive role for humoral antibodies in the therapeutic response to antimalarial drugs. The evidence that antimalarial treatment responses in immune patients were always better than those in nonimmune patients was observed first during the malaria therapy of general paralysis.[1] Since then, the phenomenon that malaria treatment responses in endemic areas improve with age has been well described. This has been interpreted as a reflection of immunity, but this has not been proved. Furthermore, the components of the immune response that are responsible for this effect have not been characterized.

In hyperendemic malarial areas such as in much of Africa, people are repeatedly exposed to infections and thereby gain a form of immunity which does not prevent all infections, but controls malaria symptoms or eliminates parasitemia spontaneously. The contributory role of antibodies in limiting malaria infections was evident even in this low-trans-

TABLE 2
Admission demographic, clinical, and antibody data at the time of admission of patients with severe and with uncomplicated malaria*

Variable	Severe malaria (n = 26)			Uncomplicated malaria (n = 54)		
	Cure (n = 13)	Failure (n = 13)	Total (n = 26)	Cure (n = 27)	Failure (n = 27)	Total (n = 54)
Age (years)†	23.8 (8.4)	24.1 (7.6)	23.9 (7.9)	23.1 (54)	23.3 (6.3)	23.2 (5.8)
PCT (hr)†	43.1 (12.2)	44.6 (8.5)	43.9 (10.3)	43.8 (8.1)‡	52.8 (16.7)	48.2 (13.7)
FCT (hr)§	37.0 (18–156)	100.0 (26–148)	50.5 (18–146)	28.0 (0–144)	37.5 (8–84)	35.0 (0–144)
IgG positive¶	13 (100)	5 (38)	18 (69)	27 (100)	19 (70)	46 (85)
IgG negative¶	0	8 (62)	8 (31)	0	8 (30)	8 (15)
IgM positive¶	7 (54)	2 (15)	9 (35)	21 (78)	10 (37)	31 (57)
IgM negative¶	6 (46)	11 (85)	17 (65)	6 (22)	17 (63)	23 (43)

* Ig = immunoglobulin; FCT = fever clearance time; PCT = parasite clearance time.
† Mean (standard deviation).
‡ Significant difference from treatment failure group ($P < 0.05$).
§ Median (range).
¶ Number (%).

mission area of Southeast Asia. In this study, patients with a previous history of malaria had higher antibody titers and lower parasite densities at admission compared with those who had their first malaria infections. This is consistent with a previous study indicating that anti-RESA antibody titers are related to exposure.[5] The finding that the proportion of patients with positive IgM at admission was higher in the uncomplicated compared with severe groups could be interpreted as indicating a possible role of IgM for controlling the severity of disease. Previous studies have also suggested a protective role for IgM antibodies against malaria infection[18,19] or disease severity.[20] The shorter fever clearance times found in patients with positive anti-RESA antibodies (IgM and IgG) at admission suggests that these and other antimalarial antibodies may have a role in the control of clinical manifestations during acute falciparum malaria.

This is in agreement with previous studies, which showed both a significant association between the presence of anti-RESA antibodies and resistance to clinical malaria,[21] and demonstrated a significant correlation between anti-RESA

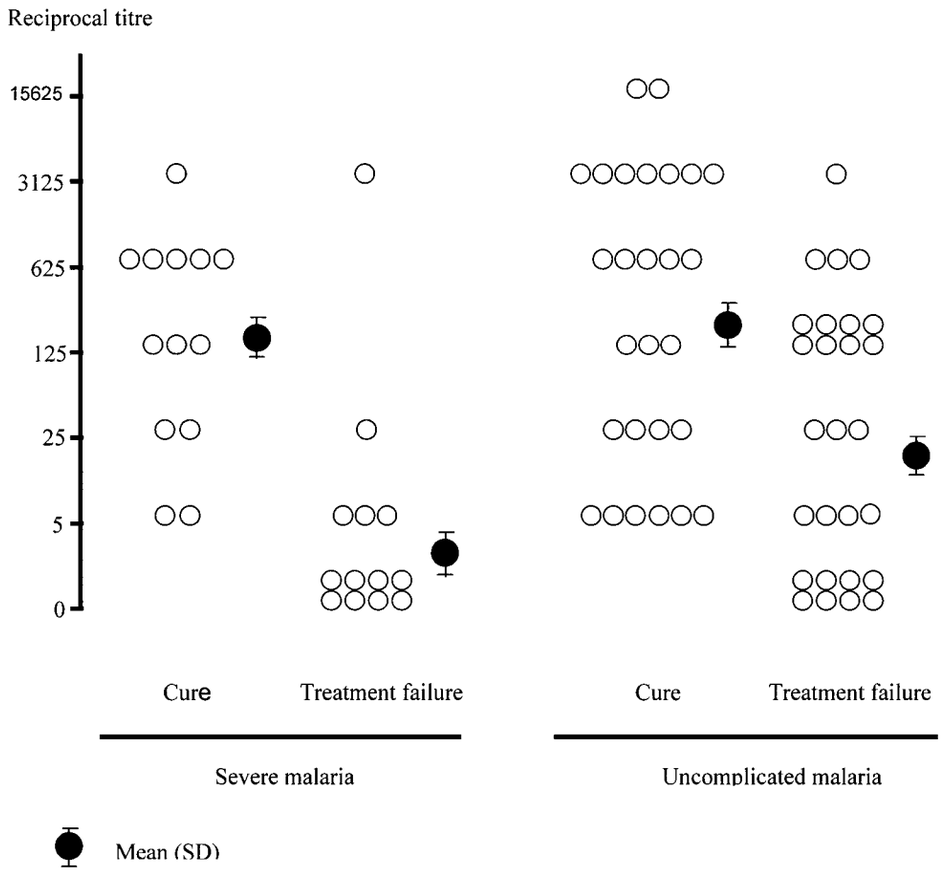

FIGURE 1. Distribution of anti-ring-infected erythrocyte surface antigen (RESA) antibody titers in patients with falciparum malaria who responded to treatment and in patients who failed to respond to treatment.

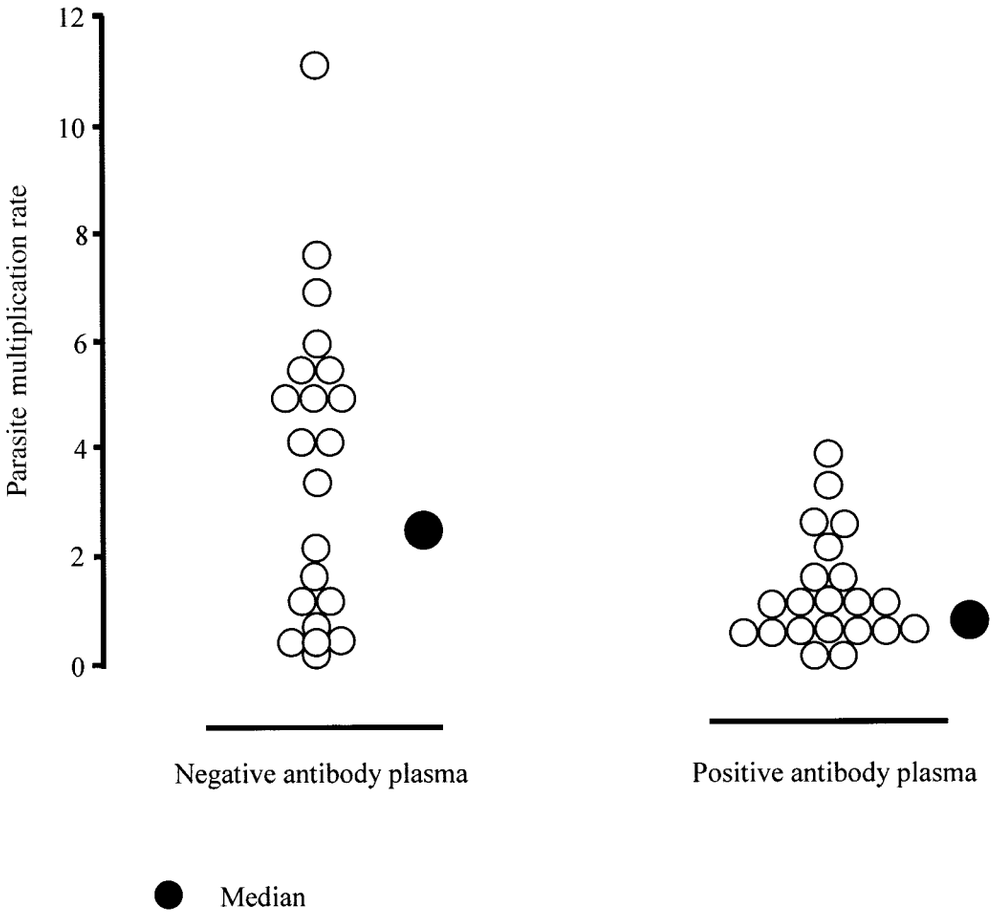

FIGURE 2. *In vitro* parasite multiplication rates in cultures containing anti-ring-infected erythrocyte surface antigen (RESA) antibody plasma and those containing normal plasma.

antibodies and low parasitemia.[5–9] It was suggested that antibodies against parasite antigens expressed in the red blood cell membrane may be of importance for controlling parasitemia. The fact that parasite clearance times were not significantly different between IgG and IgM positive and negative patients may be explained by the use of the same treatment regimens (artemisinin drugs) in both groups of patients. The parasite clearance rate achieved by these drugs far exceeds that with other drugs or host defense processes. Thus, antimalarial antibodies may not play a major part in the initial phase of parasite clearance, but their role becomes progressively more important as drug levels of the more slowly eliminated antimalarials decline. The direct contributory role of antibodies in complementing antimalarial drug activity was confirmed in this study by the inhibition of *in vitro* parasite multiplication by patient plasma containing antimalarial antibody. Although these associations and observations are suggestive, they do not prove a primary role for antibody in parasite clearance in-vivo, as antibody production may occur synchronously with activation of other arms of the host-defense system against malaria. Further studies are clearly warranted.

This phenomenon may also be relevant to the relatively faster emergence of parasite resistance to antimalarial drugs in low- compared with the high-transmission areas. In such areas antimalarial antibody, and other aspects of host-defense, may eliminate the very rare, spontaneously generated, drug resistant parasite mutants even in the absence of effective drug activity

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