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PROTECTIVE ANTIBODIES TO *PLASMODIUM FALCIPARUM* AND IMMUNITY TO MALARIA IN AN ENDEMIC AREA OF BRAZIL

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Baseline information on the prevalence of protective antibodies to malaria in endemic areas are urgently needed. These data will provide a better understanding of the process of development of resistance against plasmodia and will help to delineate strategies for programmes of vaccination against malaria. The present work showed that 19.5% of a group of 266 adults living in Ariquemes (Rondônia State, Brazil), an endemic area of malaria, presented naturally acquired antibodies to the R32 tet_{32} recombinant circumsporozoite tetrapeptide of Plasmodium falciparum. *This antibody, considered to be protective, was more frequent and showed higher titres in infected than in non-infected people. Among the population with no evidence of malaria for the last two months, 12.6% presented this antibody. The antibody was more frequently found (22.7%) in Vila Velha, the old village where most of the autochthonous population lives and among those referring a larger number of previous episodes of malaria. A second protective antibody capable of inhibiting* P. falciparum in vitro *growth was also detected in 72.4% of a small group of adults. This antibody was more frequently found in non-infected people and in those who had resided longer in the area. Our data suggest that much time is necessary for inhabitants of malaria endemic areas develop protective antibodies. The dynamics of acquisition of different protective antibodies appears to vary, possibly resulting in variability in the acquisition of protective immunity.*

It is well recognized that immunity to malaria depends on complex and ill defined mechanisms involving antibodies, other mediators and cells. The presence of protective antibodies in the blood of immune individuals has been repeatedly demonstrated by passive transfer of serum or immunoglobulin (Cohen, 1979). Methods for detecting and quantifying these antibodies *in vitro* would permit monitoring the immune status of populations exposed to the risk of malaria transmission and evaluating the protective effect of antiplasmodial vaccines. The *Plasmodium falciparum* growth-inhibition assay has been extensively used as an *in vitro* method for detecting protective antibodies to malaria (Cohen & Butcher, 1970; Jensen, Boland & Hayes, 1982; Chulay, Haynes & Diggs, 1983; Vande Waa et al., 1984).

Anti-sporozoite antibodies have been evaluated through the circumsporozoite precipitin assay and correlate with protection in immunized animals and human volunteers (Vanderberg, Nussenzweig & Most, 1969). More recently, a new assay was developed, based on the capability of antibodies to prevent invasion of sporozoites into certain tissue culture cells (Hollingdale et al., 1982, 1984). This assay has been used with several species of *Plasmodium*, and the results correlate well with protective immunity in susceptible hosts.

The tetrapeptide R32 tet_{32} from the circumsporozoite protein of *P. falciparum*, produced by genetic recombination, constitutes a candidate malaria vaccine. This tetrapeptide has been used for detecting anti-sporozoite antibodies in people living in a malaria endemic area (Hoffman et al., 1986). These antibodies have been shown to produce circumsporozoite precipitin and to inhibit sporozoite invasion of hepatoma cells, reactions which correlate with protective immunity. The use of such an antigen in an *in vitro* test would provide a useful tool for assessing the immune status of populations living in malaria endemic areas and would also help to monitor phases II and III clinical trials of malaria vaccines.

The objective of this work was to get baseline information about the prevalence of protective antibodies against *P. falciparum* sporozoites and erythrocytic forms in a malaria endemic area of Brazil. These data will help us to understand the process of development of resistance against malaria and to plan strategies for programmes of vaccination in endemic areas.

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MATERIALS AND METHODS

Study population — This study comprised 266 individuals living in the city of Ariquemes (Rondônia, Brazil) and selected through a clinico-epidemiological survey during the period of June-July, 1985. In 84 of those individuals (31.5%) malaria was diagnosed by means of blood smears by the local unit of SUCAM (Ministry of Health, Brazil). Their ages ranged from 14-65 years ($\bar{x}$ = 36.5 ± 14.4), and 76.2% were males. Then non-infected group comprised 182 individuals with negative blood smears and referring no symptoms of malaria for at least two months previously. Ages ranged from 14-73 years ($\bar{x}$ = 35.4 ± 15.4) and 54.4% were females. All study cases formally agreed to participate in the investigation and to donate 5-10ml of blood. Children below 14 years old were excluded from the study.

The city of Ariquemes has experienced an explosive increase in population, starting in 1975, due to the arrival of thousands of migrants, mainly from the non-endemic areas of south Brazil. They occupy rural settlements around the city and live from agricultural activities. The population in 1985 was over 110,000 inhabitants, which represented an increase of 43.2% compared to the previous year. Cases of malaria occur throughout the year, with peaks usually during May to July. In 1985 the annual parasite incidence index, measured as the number of cases per 1,000 inhabitants, was 268, while the spleen index was 9.4% among 2 to 9 year old children, and 9.0% in the adult population. *P. falciparum* was responsible for 41.1% of the cases of malaria (Moura et al., 1986). Sixty-nine percent of the population examined (1,208 individuals) referred previous malaria, most of them (83.0%) suffering multiple episodes.

Blood — Blood was collected by venipuncture in Vacutainer tubes and allowed to clot at room temperature. Serum was collected and sent at 4°C by air to the Laboratory of Malaria, Universidade de Brasília. Processing of serum samples consisted of aliquoting and storage at –70°C until use in ELISA tests. Alternatively, sera were dialyzed for 48hrs. at 4°C (cut off 10-12,000 daltons) in 0.9% NaCl solution (1:1,000), followed by medium RPMI 1640 (1:10), inactivation at 56°C for 30 min, and sterilization by filtration through 0.45μm cellulose acetate membrane. These latter sera were used for parasite growth inhibition assays.

Enzyme linked immunosorbent assay (ELISA) for detection of antibodies to circumsporozoite protein — The antigen used was the tetrapeptide R32 tet_{32} produced by genetic recombination in *Escherichia coli* by Smith, Klein & French (supplied by Dr. Robert A. Wirtz, Dept. of Entomology, Walter Reed Army Institute of Research, Washington). It consisted of 30 asn-ala-asa-pro repeats plus 2 asn-val-asp-pro repeats fused to a 32 amino acid protein.

Immulon 2, "U" 96 well plates (Dynatech Laboratories, Alexandria, USA) were sensitized overnight at room temperature with 0.1μg of antigen/well, and the remaining active sites on the plate blocked for 1hr. with a solution of 0.5% boiled casein (Baker Chemical, E 397-7) in phosphate buffered saline (PBS), pH 7.4, plus 1% Tween 20. Serum samples were diluted to 1:100 in a solution of 0.5% boiled casein plus 0.025% Tween 20, and 50μl added to each well in triplicate, followed by 2hrs. incubation at room temperature. Wells were washed two times with 0.05% Tween 20 in PBS pH 7.4, and 50μl of peroxidase conjugated anti-human IgG (Miles-Yeda, 61-231) diluted to 1:2,000 in 0.5% boiled casein plus 0.025% Tween 20, added to each well. After 1 hr. incubation at room temperature, plates were washed three times, and 100μl of peroxidase substrate (2, 2'-azino-di-(3-ethyl-benzthiazoline sulfonate)) ABTS + H_2O_2 (Kirkegaard & Perry, 50-62-00) was added to each well, and the plates were incubated for 30 min at room temperature. Color development was read at 405nm in an ELISA plate reader spectrophotometer (Titertek-Multiskan, Dynatech Laboratories) connected to an IBM microcomputer. The ELISA protocol followed was essentially that suggested by Dr. R.A. Wirtz (WRAIR, Washington, D.C.). Sera were considered to be positive for antibody to the R32 tet_{32} tetrapeptide when the mean absorbance of triplicate blank wells containing no antigen was greater than the mean plus two standard deviations of the absorbance of 30 negative control sera from blood donors with no previous history of malaria. Positive and negative control sera were run in each plate.

Plasmodium falciparum **growth inhibition assay** — The *P. falciparum* isolate # 144, collected from a patient of Ariquemes (Rondônia State, Brazil), and adapted to *in vitro* growth, was used after synchronization by two treatments with sorbitol solution, as described by Vernes et al. (1984). Seventy-two hours after the second sorbitol treatment, when nearly 90% of the parasites were in the early schizont stage, the culture was diluted with normal O^+ erythrocytes to a parasitemia of 1% and adjusted to 4% hematocrit with Hepes-buffered (20mM) RPMI-1640 medium

(GIBCO), containing 10% of a pool of 50 normal human serum, 2mM glutamine, 25mg/ml gentamycin, and 0.2% NaHCO. [3H]-hypoxanthine, specific activity > 11mCi/mmol (Amersham) was added to the suspension to give a concentration of 0.5μCi/100μl/well, and 50μl/well of test and control sera added in triplicates to the plates. After incubation at 37°C for 48hrs. in a wet chamber with a mixture of 5% CO_2 in air, parasites were collected in glass fiber strips with a cell harvester and radioactivity read in a Beckman beta scintillation counter. The percentage of growth inhibition was calculated according to the formula:

$$\% \text{ inhibition} = 100 - \frac{\textit{cpm in the presence of test serum}}{\text{cpm in the absence of test serum}} \times 100$$

An inhibition of growth equal to or higher than 30% was considered as positive.

Statistical analysis – Statistical tests used were two tailed independent Student's *t*-test, Chi square (with Yate's correction), and Pearson's test of correlation of two variables.

RESULTS

Antibodies to the R32 tet_{32} circumsporozoite protein of *P. falciparum* were significantly more frequent and showed higher levels in malaria infected individuals, as compared to non-infected people living in the same endemic area (Table I). Only 12.6% of 182 non-infected individuals presented anti-sporozoite antibodies, while 34.5% of the 84 presenting patent parasitemia gave a positive reaction.

TABLE I

Positive ELISA to anti-R32 tet_{32} antibodies in *P. falciparum* infected and non-infected individuals living in Ariquemes.

Group	Frequency of Positive Reactions (*)	(%)	Mean Absorbance (**) ± SD
Infected	*29/84*	*(34.5)*	0.322 ± 0.380
Non-Infected	23/182	(12.6)	0.157 ± 0.191
Total	52/266	(19.5)	0.225 ± 0.315

(*) $p < 0.001$; (**) $p < 0.01$.

To investigate whether the frequency of positive anti-R32 tet_{32} antibody reactions was uniformly distributed in the population studied, infected people were distributed into four different groups, according to the area they lived and the epidemiological conditions for malaria transmission.

As shown in Table II, the frequency of positive reactions varied widely depending on local of residence in Ariquemes. Those living in Vila Velha, a small village from which Ariquemes grew and where most of the autochthonous population is concentrated, showed the highest frequency (22.7%) and mean antibody level (0.1859), while those living in the Burareiro Project, *consisting of large rural settlements*, presented the lowest frequency of positive reactions.

TABLE II

Positive ELISA to anti-R32 tet_{32} antibodies in people living in different localities of Ariquemes.

Locality	Distribution	Frequency of Positive Reactions (*)	(%)	Mean absorbance (**) ± SD
Vila Velha	peri-urban	10/44	(22.7)	0.1859 ± 0.2338
Chácaras	peri-urban	1/16	(6.3)	0.0542 ± 0.0991
Marechal Dutra	rural	11/93	(11.8)	0.0842 ± 0.1917
Burareiro	rural	1/29	(3.4)	0.0655 ± 0.1117

(*) $p < 0.05$; (**) $p < 0.001$.

Although males showed a higher frequency and level of anti-R32 tet_{32} antibodies than females (Table III), as could be antecipated from their professional activities, no statistical significance was found between the two groups. However, a significantly higher frequency of anti-circumsporozoite tetrapeptide antibodies was found in individuals arriving from other malaria endemic areas, as compared to that of people coming from non-endemic regions (Table IV).

TABLE III

Sex distribution of positive ELISA for anti-R32 tet_{32} antibodies from people living in Ariquemes.

Sex	Frequency of Positive Reactions (*)	(%)	Mean Absorbance (**) ± SD
Male	14/83	(16.9)	0.1319 ± 0.2374
Female	9/99	(9.1)	0.0790 ± 0.2292

(*) $p < 0.12$; (**) $p < 0.13$.

TABLE IV

Frequency and levels of anti-R32 tet_{32} antibodies detected by ELISA in individuals living in Ariquemes, according to their origin in endemic or non-endemic areas of malaria.

Origin	Frequency of Positive Reactions (*)	(%)	Mean Absorbance (**) ± SD
Non-Endemic Areas	13/139	(9.4)	0.0871 ± 0.2356
Endemic Areas	10/43	(23.3)	0.1552 ± 0.2228

(*) $p < 0.01$; (**) $p < 0.1$.

During the clinico-epidemiological survey, people were asked how many episodes of malaria they had previously experienced. A positive correlation was found between the number of episodes of malaria reported and the frequency and level of anti-R32 tet_{32} antibodies (Table V and Fig. 1). In addition, people with a positive reaction had an average of 21.6 ± 24.3 previous episodes, while those with negative reactions had an average of 9.4 ± 16.0 episodes.

TABLE V

Antibodies to the R32 tet_{32} tetrapeptide detected by ELISA and number of previous episodes of malaria in individuals living in Ariquemes.

Number of Episodes	Frequency of Positive Reactions (*)	(%)	Mean Absorbance (**) ± SD
0	1/47	(2.1)	0.0086 ± 0.0877
1 to 9	8/76	(10.5)	0.0990 ± 0.1398
10 to 19	3/18	(16.7)	0.0604 ± 0.1828
20 or more	11/41	(26.8)	0.2391 ± 0.2405
Total	23/182	(12.6)	0.1570 ± 0.1913

(*) $p < 0.05$; (**) $p < 0.001$.

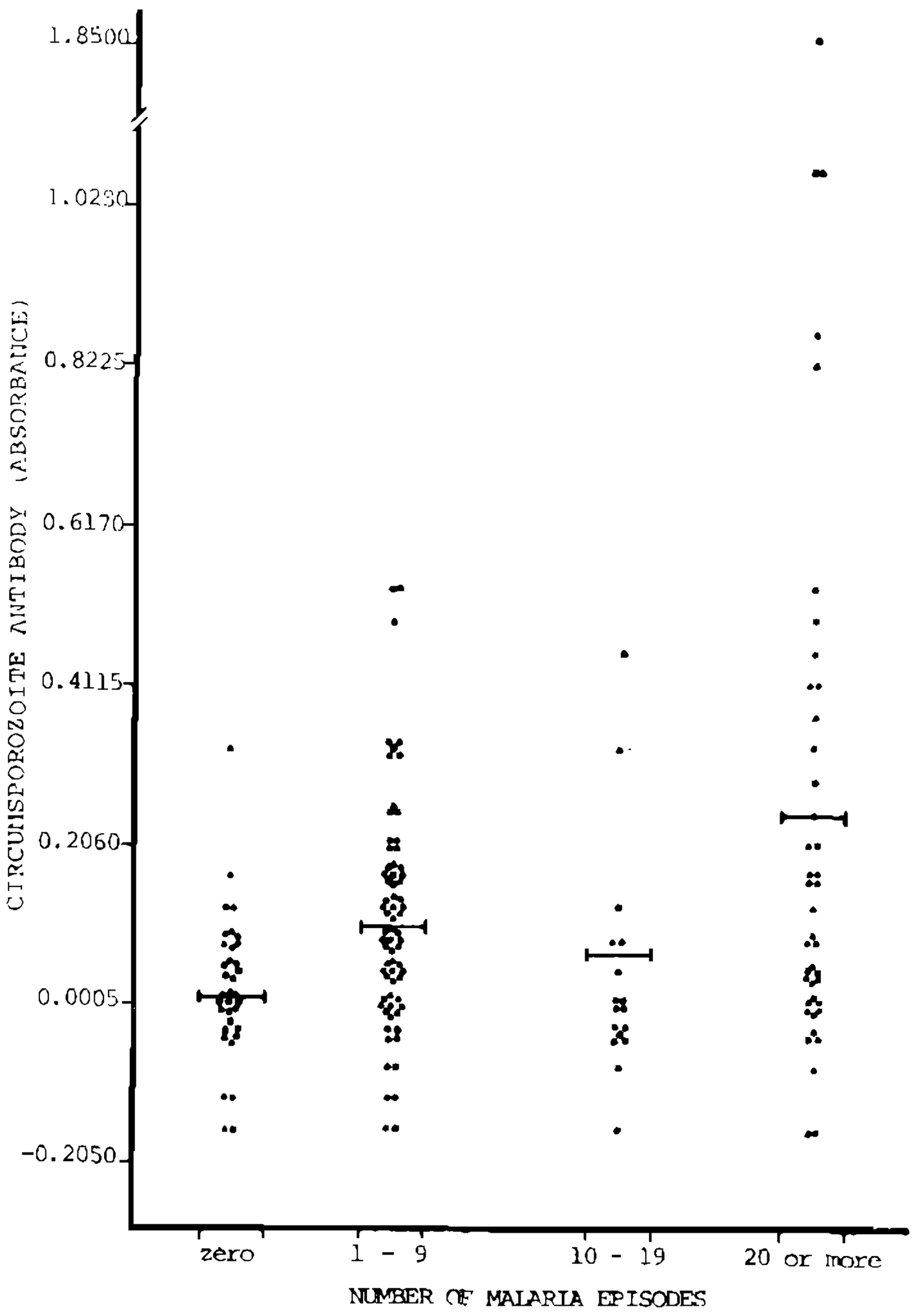

Fig. 1: Relationship between number of previous episodes of malaria and levels of anti-R32tet_{32} antibodies, detected by ELISA, and represented by absorbance at 405 nm. Bars represent mean absorbance of each group.

The frequency of growth inhibition antibodies among the non-infected group was higher than that found in infected people (Table VI). The proportion of people with growth inhibiting antibodies in the different localities of Ariquemes did not show any significant difference, nor did the proportion vary according to the origin of the individuals from malaria endemic or non-endemic areas (data not shown).

TABLE VI

Presence of *P. falciparum*-growth inhibiting antibodies in infected and non-infected people living in Ariquemes.

Group	Frequency of Growth Inhibition (%)						Positive Reactions (%)
	<30%		30 - 60%		>60%		
Infected	6/17	(35.3)	10/17	(58.8)	1/17	(5.9)	64.7
Non-Infected	2/12	(16.7)	8/12	(66.7)	2/12	(16.7)	83.4
Total	8/29	(27.6)	18/29	(62.1)	3/29	(10.3)	72.4

An apparent relationship was found between the level of growth inhibition antibody and time living in the endemic area: people giving a negative reaction had lived in Ariquemes for an average of 9.2 years, while those with growth inhibitory antibodies had lived there for an average of 13.2 years. Interestingly, the highest degree of inhibition (> 60%) was found among people living in the endemic area for an average of 15.4 years, and referring an average of less than one previous episode of malaria (Table VII).

TABLE VII

Relationship between *P. falciparum*-growth inhibiting antibodies and time living in malaria endemic area and number of previous malaria episodes

Percentage of Growth Inhibition	Number of Individuals	Time Living in Endemic Area (years) Mean ± SD	Previous Episodes of Malaria Mean ± SD
< 30	8	9.2 ± 12.9	7.7 ± 7.9
30 – 60	18	12.8 ± 13.4	9.2 ± 13.7
> 60	3	15.4 ± 13.5	0.3 ± 0.6

DISCUSSION

Studies on the acquisition of immunity to malaria by populations exposed to transmission and the nature of the process pose several difficulties: 1) the inexistence of a simple and reliable technique for assessing protective immunity in field conditions; 2) the apparent lack of correlation between the results of *in vitro* tests and parameters of clinical immunity; 3) the features of immunity to malaria; i.e., it requires a longstanding contact with the parasite, it is not usually complete, and it needs a constant antigenic stimulus to be maintained. These difficulties explain the scarcity of data on this subject. The recent development of techniques capable of detecting antibodies possessing a putative protective effect has opened a new avenue, allowing one to answer important questions on the immune status of populations living in malaria endemic areas. Two of such techniques have been used in this work: detection of antibody directed towards the R32 tet_{32} circumsporozoite tetrapeptide of *P. falciparum*, and detection of an antibody capable of inhibiting *in vitro* growth of *P. falciparum*. Although the detection of antibodies both to sporozoites and asexual erythrocytic forms, as used in our work, represented an attempt to get a more comprehensive picture of the nature of the immune response, this study had the following drawbacks: a) the detection of anti-circumsporozoite antibody was not based on a functional assay; b) although tested through a functional assay, growth inhibition antibodies may not always correlate with protection *in vitro* (Perlmann, 1986), or the inhibition may not be due to the presence of immunoglobulins in the serum (Tharavanij et al., 1984); and c) both antibodies detected are species-specific, meaning that immunity to *P. vivax* was not considered.

The antigen used to detect anti-circumsporozoite antibodies was the R32 tet_{32} tetrapeptide produced by genetic recombination in *E. coli* (Young et al., 1985). This peptide has been shown to elicit antibodies in animals which block sporozoite invasion of hepatoma cells (Young et al., 1985), suggesting that it can be used for detecting protective antibodies. Indeed, recently Hoffman et al., 1986 have demonstrated that people living in a malaria endemic area produced anti-R32 tet_{32} antibodies, and that fraction IgG and IgM, purified from these antibodies, had the ability to inhibit sporozoite invasion of a human hepatoma cell line. Thus, detection of antibodies to R32 tet_{32} by means of ELISA, as used in our study and not necessarily by a functional assay, is considered to be an *in vitro* correlate of protective immunity.

Our data showed that 19.5% of a group of 266 adults living in Ariquemes, an endemic area for malaria, possessed antibodies to the R32 tet_{32} tetrapeptide, and that both the frequency and the levels of these antibodies were higher in infected people. Considering that the vast majority of these people are diagnosed and treated for malaria attacks very soon after infection, one may suggest that anti-R32 tet_{32} antibodies rise soon after the antigenic stimulus represented by exposure to sporozoites, and, once stimulus ceases, the level of antibody drops. However, the finding that the frequency and levels of antibody increase with time living in the endemic area (Moura & Tosta, in preparation) and with the number of previous episodes of malaria suggests that a certain degree of immunity to the sporozoite is gradually being acquired by the population exposed to *infection, and that each reinfection may act as a booster.*

We showed that the presence of anti-R32 tet_{32} antibodies varied with the area were people lived. The frequency of this antibody in individuals living in Vila Velha, for instance, was 6.7 x higher and the average level of antibody was 2.8 x higher than that of people living in Burareiro. Two possible explanations could be suggested. Vila Velha was the small village, inhabited since 1916, from which Ariquemes grew nearly 50 years later. Presently, it represents a district of Ariquemes, located in the peri-urban area, where most of the autochthonous population still live. The longstanding contact of this population with malaria probably allowed them to develop a higher degree of immunity. This immunity could have been boosted very often by frequent reinfection, since the Anopheles density in Vila Velha is very high (Moura, in preparation). Burareiro, on the other hand, was the first project of colonization to be settled in the rural area of Ariquemes. People live on medium sized farms, and prevalence of malaria is lower than in the other areas. People lived in Burareiro for an average of 4.0 years, while in Vila Velha the average was 12.0 years. In contrast to the low prevalence of anti-R32 tet_{32} antibodies in people living in Burareiro, those from Marechal Dutra, another rural settlement, presented a significantly higher prevalence. Difference of epidemiological conditions may help to explain these results, since in the latter area colonization has occurred more recently; the population is mainly from non-endemic areas, is less stable and lives in smaller rural settlements; and transmission of malaria is higher (Moura, in preparation).

Our data suggest that *in vitro* growth inhibition antibodies possess a dynamics different from anti-R32 tet_{32} antibodies. They appear to be more frequent in non-infected people from the endemic area and may not directly correlate with the number of previous malaria episodes (see Table VII). They appear to increase with time living in the endemic area. However, our study group is not large enough to allow us to draw reliable conclusions.

Our results demonstrated that, in a population represented mostly by non-immune people, much time elapses before the acquisition of protective immunity. The results further suggest that this immunity is reinforced by the frequent boosters of reinfection, and that studies of the dynamic of acquisition of immunity to malaria give variable results depending on the protective antibody studied.

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